

Pickled Vegetables Intake Impacts the Metabolites for Gastric Cancer

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Purpose: An increased risk of gastric cancer (GC) for pickled vegetables intake has been suggested, but a complete understanding of its pathogenic origin is still lacking, especially from a metabolic viewpoint. We investigated the plasma metabolites and metabolic pathway alteration of GC related to pickled vegetables intake.

Methods: We analyzed plasma samples collected from 365 gastric cancer patients and 347 healthy individuals, and divided them into three subgroups according to the intake of pickled vegetables. Plasma samples were detected by untargeted metabolomics.

Results: Nine metabolites were significantly altered in GC patients among pickled vegetables intake groups (FDR P -value<0.05). All of them were associated with the risk of gastric cancer adjusted for gender, age, smoking status, *Helicobacter pylori* infection. Pathway analysis showed significant alteration in the folate biosynthesis pathway.

Conclusion: In short, we provide new insights from a metabolic perspective on the relationship between pickled vegetables intake and the occurrence of gastric cancer.

Keywords: pickled vegetables intake, metabolites, metabolic pathway, gastric cancer

Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide.¹ However, the occurrence of GC varies from place to place, with "Eastern Asia Countries" like the People's Republic of China, Korea, and Japan experiencing the highest incidences while North America and Africa experience the lowest. Recent evidence shows that the GC pattern of most Chinese American immigrants is more consistent with their new country of residence than with their country of origin.² Given this geographical variation, environmental and dietary factors may play more important roles than genetic factors in gastric oncogenesis. Therefore, there are growing interests in studying the impact of dietary changes on GC incidence.

Vegetable consumption is known to contribute to the reduction in GC risk.³ However, the incidence rates of GC remain high in Chinese, Japanese, and Korean populations, even though they have a high consumption of total vegetables.^{4,5} This may be due to the fact that they mainly consume pickled vegetables, which were the traditional and important part of their dietary culture. Several studies have shown that pickled vegetable intake could increase the risk of GC.^{4,6,7} It has been shown that the risk of GC for pickled vegetables intake is even higher than *Helicobacter pylori* (*H. pylori*) infection among Chinese populations (OR:1.49 [95% CI:1.10–2.01] vs 1.26 [95% CI: 1.12–1.62]).⁸ Although several mechanisms have been

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postulated in epidemiological studies,^{9–11} the molecular mechanism of pickled vegetable intake in gastric carcinogenesis remains unclear.

Metabolomics, as the end point of the signaling cascade, represents the final response of living systems to environmental, genetic, and disease factors, and is routinely applied as a tool for biomarker discovery.^{12–14} For example, Lario et al¹⁵ found that histidine, tryptophan and phenylacetylglutamine could possibly be used as potential GC biomarkers between non-GC and GC groups.¹⁵ The correlation between metabolomics and phenotypes enables metabolites to easily model pathophysiological changes in the molecular level in cancer. Due to innovative developments in bioinformatics and analytical technologies, metabolomics analysis is now expanded to provide insight into the mechanisms that underlie various physiological conditions and aberrant processes.¹⁶ For example, metabolomics was used to further study the potential molecular mechanism of gastric cancer caused by *H. pylori*.¹⁷ Therefore, metabolomics is a great tool to study the underlying molecular mechanism of gastric cancer caused by pickled vegetables intake.

In this study, we set out to examine the potential effects of pickled vegetables intake by measuring a broad range of blood metabolites and examined their correlation with gastric cancer. We applied untargeted metabolomics to plasma samples obtained from 365 GC patients and 347 healthy individuals.

Methods

Study Population

This study was based on a population-based case-control study, in which GC patients and healthy individuals were consecutively enrolled in Xianyou County between March 2013 and December 2017. All newly diagnosed cases of GC which were confirmed histologically based on tissue specimens and who had lived in Xianyou for at least 10 years were included. GC patients with other cancers, secondary or recurrent gastric cancer, gastritis, previously received neo-adjuvant chemotherapy or chemoradiotherapy or radiotherapy, pregnant, metabolic diseases such as diabetes, gout, hyperlipidemia, systemic administration of corticosteroids, neurological and psychiatric diseases, severe hepatic and renal dysfunction; and severe respiratory disease requiring continuous oxygen treatment, etc, were excluded. For the healthy individuals, there was no abnormality of blood tests and

endoscopic examinations. A total of 365 GC cases and 347 healthy controls were recruited, and based on the median intake of pickled vegetables of subjects 10 years ago, all of them were divided into three subgroups: the group of no pickled vegetable intake 10 years ago (NPVI = 0g/week) (62 GC cases and 76 healthy controls), the group of low level pickled vegetable intake 10 years ago (LPVI \leq 350 g/week) (133 GC cases and 110 healthy control) and the group of high level pickled vegetable intake 10 years ago (HPVI >350 g/week) (170 GC cases and 161 healthy controls). This study was approved by the Biomedical Research Ethics Committee of Fujian Medical University, China (no.97,2014), in line with the relevant regulations and requirements of medical ethics. Written consents were obtained from all participants at study enrollment.

Questionnaire Data Collection

Face-to-face interviews were conducted by trained investigators. A structured questionnaire was applied to collect information on demographic features, lifestyles, family cancer history, cigarette smoking and alcohol consumption, which has been described previously.⁸ It is noteworthy that a food frequency questionnaire (FFQ) was also used to obtain participants' dietary habits and food intake, including the frequency and amount 10 years before diagnosis in the GC cases and 10 years before interview in the controls. For each kind of food, participants were asked to describe how often (per day, per week and per month) and the serving size they had. We made an estimation of average pickled vegetable intake per week for each subject, expressed as the product of frequency per week and usual serving size in grams. A commonly used serving bowl and some food pictures were presented to help subjects recall their past consumption of specific food when investigators collected dietary information. The study was introduced to the participants as a diet and health study in order to reduce recall bias.

Sample Collection and Preparation

All the study participants were in an overnight fasting state and 5 mL of peripheral venous blood was taken in the morning. The blood was then allowed to clot for 30 minutes at 37°C water bath and followed by centrifugation at 3000 rpm for 10 minutes, then was divided into plasma, white blood cells, and red blood cells and stored at -80°C until further analysis.

Plasma samples were thawed on ice, 300 μ L of cold acetonitrile were added to 150 μ L of plasma, vortexed and

kept at 4°C for 30 min, 150 µL of cold acetonitrile were added again and vortexed and kept at 4°C for 3 hours, for protein precipitation. After centrifugation at 16,000 g (30 min, 4°C) and 500 µL supernatant filtration with 0.22 µm filter, 100–150 µL resulting supernatant were transferred to liquid chromatography-mass spectrometry (LC-MS) vials and stored at –20°C until subsequent LC-MS analyses. Quality control (QC) was prepared with standard plasma sample.

Metabolic Profiling

Each sample was processed by Agilent 1200 high performance liquid chromatography combined with a 6520 accurate electrospray ionization/quadrupole-time-of-flight mass system (Agilent Technologies, CA, USA). Plasma samples were separated on an Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm, Agilent Technologies), with the condition of 150 µL injection volume, 0.25 mL/min flow rate and 37°C column temperature, by using a gradient program of the mobile phase A 0.1% formic acid solution (ESI+)/water (ESI-) and mobile phase B was acetonitrile with 0.1% formic acid solution (ESI+)/acetonitrile (Merck, Darmstadt, Germany) (ESI-). The gradient program started from 2% B for 0–9 minutes, linearly increased from 2 to 60% B for 9–18 minutes, linearly increased from 60 to 100% B for 18–20 minutes, and kept at 100% B for 20–30 minutes. To avoid cross contamination from plasma samples, plusing an ultra-pure water elution after each sample. All the data were collected in ionization quadrupole time-of-flight mass spectrometry with both positive (ESI+) and negative (ESI-) full scan mode. The parameters of mass spectrometry were as following: GAS1, 80; GAS2, 70; CUR, 40; TEM, 375°C; and ISVF, 5500 V; a mass range of 50 to 1500; scan time of 0.3 seconds.

Data Processing and Statistical Analysis

mzData files were converted to the mzXML format using Agilent MassHunter qualitative analysis software and processed using XCMS online (<https://xcmsonline.scripps.edu>), which performed feature detection, retention time correction, alignment, annotation, etc. Metabolic peaks detected less than 80% in all QC samples were discarded.¹⁸ Identification of metabolites was carried out by matching m/z (Da) values against the Human Metabolome Database (HMDB, <http://www.hmdb.ca>).

Prior to statistical analysis, each metabolic peak in all subject samples was normalized based on QC samples for removing the unwanted analytical variations occurring

intra- and inter-batches. And the plasma abundant values of metabolites investigated were set to a log scale and auto-scaled (mean-centered and divided by the standard deviation of each variable) using MetaboAnalyst 4.0. An orthogonal partial least squares discriminant analysis (OPLS-DA) and univariate two-sided *t*-test were used for metabolic profile description and metabolic signature discovery between GC cases and healthy controls among the NPVI, LPVI and HPVI groups, respectively. The false discovery rate (FDR) method was used to correct for multiple hypothesis testing and to reduce false positives. Those metabolic features with variable importance in the projection (VIP) values >1.0 in the OPLS-DA model and FDR adjusted *p*-values < 0.05 in the *t*-test were considered to be significantly different between GC cases and healthy controls. Then, using a venn diagram we selected the discriminant metabolites associated with pickled vegetables intake. Metabolites in groups LPVI and HPVI but outside the overlap part with group NPVI were considered to be related to pickled vegetables intake. The boxplots were used for depiction of highly discriminant metabolites related to intake of pickled vegetables between GC cases and healthy controls in the LPVI and HPVI groups. Subsequently, odds ratio (OR) and 95% confidence intervals (95% CI) for the association between metabolites related to pickled vegetables intake and GC risk were determined by logistic regression. Finally, pathway analysis was run with MetaboAnalyst 4.0.

Results

Participant Characteristics

A total of 365 GC cases and 347 healthy controls were included, with a mean age of (65.15±9.03) years and (64.95±10.07) years, respectively. As shown in Table 1, there was no statistically significant difference in gender, age, smoking status, *H. pylori* infection, among the three subgroups (*p*>0.05), except a significant difference in gender among the LPVI group (*p*<0.05).

Metabolic Profiles of Plasma Samples

A total of 225 compounds were extracted from the LC-MS data. After being excluded based on Extracted Ion Chromatogram (EIC), 131 compounds were selected for subsequent analyses. After structurally identified by comparing with data shown in HMDB (www.hmdb.ca) databases, 51 compounds were identified from seven chemical classes, of which 14 were lipids, 17 were nucleotides, 4

Table 1 Demographic Characteristics of Study Participants

| | Total (n=712) | | NPVI (n=138) | | LPVI (n=243) | | HPVI (n=331) | |
|----------------------------|---------------|---------|--------------|---------|--------------|---------|--------------|---------|
| | Case/Control | p-value | Case/Control | p-value | Case/Control | p-value | Case/Control | p-value |
| Gender | | | | | | | | |
| Male | 271/265 | 0.512 | 53/60 | 0.321 | 95/94 | 0.009 | 123/111 | 0.496 |
| Female | 94/82 | | 9/16 | | 38/16 | | 47/50 | |
| Age (years) | | | | | | | | |
| ≤65 | 194/187 | 0.843 | 39/44 | 0.55 | 81/64 | 0.667 | 74/79 | 0.312 |
| >65 | 171/160 | | 23/32 | | 52/46 | | 96/82 | |
| H. pylori infection | | | | | | | | |
| Yes | 143/123 | 0.304 | 19/28 | 0.445 | 59/37 | 0.089 | 65/58 | 0.677 |
| No | 222/224 | | 43/48 | | 74/73 | | 105/103 | |
| Smoking | | | | | | | | |
| Yes | 198/189 | 0.953 | 33/41 | 0.933 | 76/67 | 0.553 | 89/81 | 0.71 |
| No | 167/158 | | 29/35 | | 57/43 | | 81/80 | |

Abbreviations: NPVI, no pickled vegetable intake 10 years ago; LPVI, low pickled vegetable intake 10 years ago; HPVI, high pickled vegetable intake 10 years ago.

were amino acids, 4 were peptides, 5 were carbohydrates/other, 5 were cofactors/vitamins, and 2 were energy pathway metabolites (Figure 1A). OPLS-DA score plots

showed the differences between GC patients and healthy individuals in the three groups, suggesting variations in their metabolomes (Figure 1B–D).

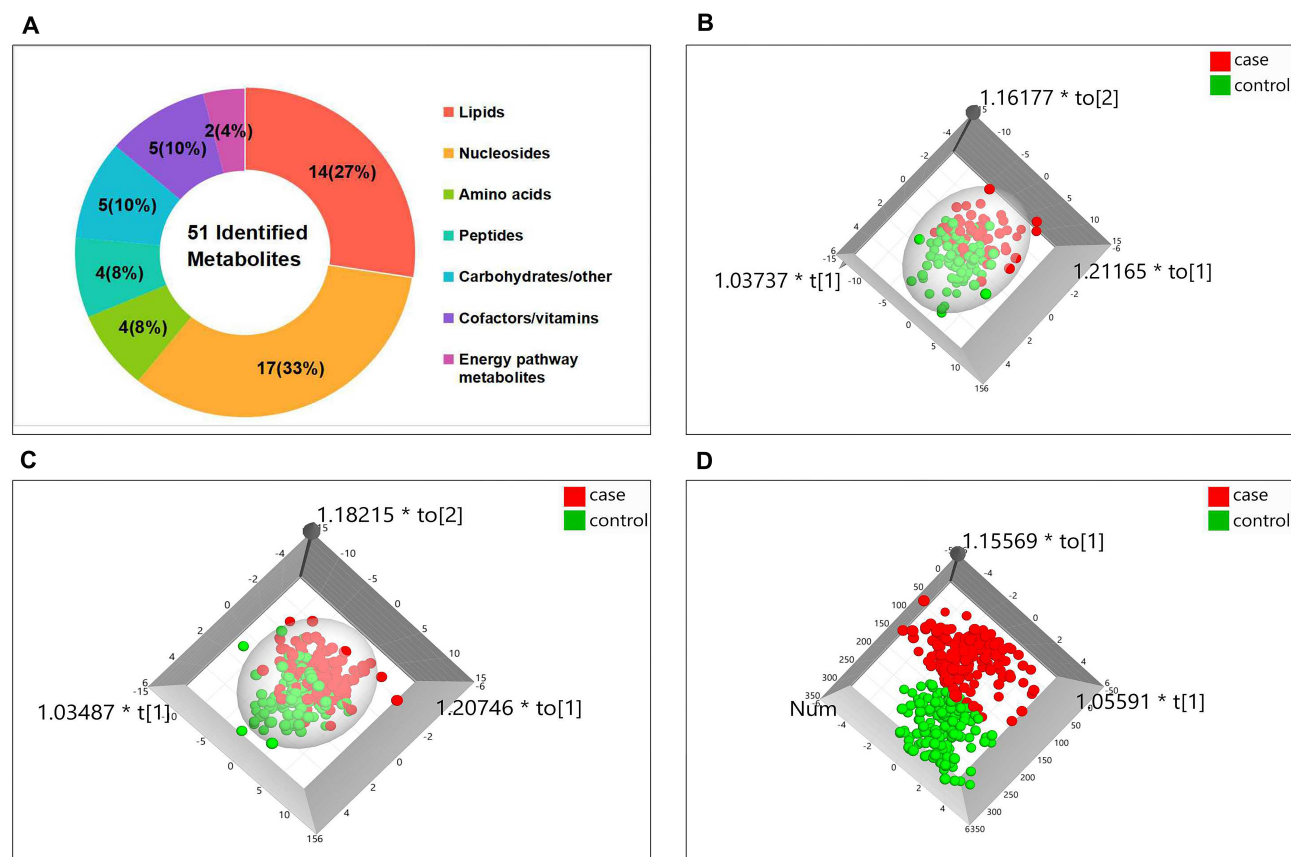


Figure 1 Metabolic profiles of plasma samples. (A) Total identified metabolites from plasma of GC cases and healthy controls. Orthogonal partial least-squares discrimination analysis (OPLS-DA) score plots in the groups NPVI (B), LPVI (C), and HPVI (D).

Changes in Plasma Metabolites Associated with Pickled Vegetables Intake

To find metabolites associated with pickled vegetables intake, comparisons were made between GC cases and healthy controls among the NPVI, LPVI, and HPVI groups. First, univariate two-sided *t*-test and the variable importance in projection (VIP) scores from OPLS-DA models were carried out to identify different metabolites between GC patients and healthy individuals, of which 8 were different in the NPVI group, 14 in the LPVI group, and 14 in the HPVI group. Metabolites with FDR adjusted *p*-values < 0.05 and VIP > 1 were selected as discriminants. Next, a venn diagram was used to find the important metabolites associated with pickled vegetables intake (Figure 2A). Eleven metabolites, which were in groups LPVI and HPVI but outside the overlapping part with group NPVI, were regarded as the changed metabolites related to pickled vegetables intake. They are glutathione, paraxanthine, L-histidinol, guanosine triphosphate, testosterone, N-acetylmuramate, porphobilinogen, guanosine, dihydrobiopterin, cytidine triphosphate, and 11-trans-leukotriene C4. Among them, the unique metabolites for groups LPVI and HPVI might be due to the intake of pickled vegetables. Figure 2B shows boxplots of relative abundance of changed metabolites related to pickled vegetable intake in the LPVI and HPVI groups. The change trend of metabolites related to pickled vegetable intake in the overlapping parts of LPVI and HPVI is the same, suggesting that the relative abundance of testosterone, dihydrobiopterin, and guanosine were increased in the GC group. On the contrary, guanosine triphosphate, N-acetylmuramate and porphobilinogen were decreased, compared with the control group. The difference of metabolites between GC cases and healthy controls is statistically significant (FDR *p* < 0.05). To further certify that the metabolites change in plasma is related to the intake of pickled vegetables, we compared the relative abundance of plasma metabolites among subjects of NPVI, LPVI, and HPVI in the controls and the cases, respectively. Figure 2C shows that the changing trends of metabolites among NPVI, LPVI, and HPVI in the GC cases and the controls were similar. Only three metabolites (glutathione, paraxanthine, and 11-trans-leukotriene C4) were statistically significantly different between LPVI and HPVI in GC cases (*p* < 0.05). However, except for porphobilinogen and L-histidinol, the relative abundance of plasma metabolites between NPVI and HPVI in cases or in controls was

statistically different (*p* < 0.05), suggesting that nine metabolites were associated with pickled vegetables intake.

Association Between Metabolites Related to Pickled Vegetables Intake and Gastric Cancer

Figure 3 presents odds ratios for the association between metabolites related to pickled vegetables intake and GC. We presented the results in three groups: groups LPVI and HPVI were adjusted for gender. Total subjects including NPVI, LPVI and HPVI were adjusted for gender, age, smoking status, and *H. pylori* infection. Results from the three groups were similar, and the confidence intervals for the associations reported in the total subjects are notably narrower. Metabolites including testosterone, dihydrobiopterin, guanosine, paraxanthine, glutathione, cytidine triphosphate, and 11-trans-leukotriene C4, showed that their relative abundance was increased in the GC group, were associated with increased risks of GC. Conversely, metabolites including guanosine triphosphate and N-acetylmuramate, with a decreased relative abundance, were associated with a decreased risk of GC. In short, metabolites related to pickled vegetables intake are associated with the occurrence of gastric cancer.

Pathway Analysis of Altered Metabolites Related to Pickled Vegetables Intake Between GC Cases and Healthy Controls

The nine altered metabolites related to pickled vegetables intake were pooled into pathway analysis, which was used to extract biological information within relevant networks of metabolic pathways integrating metabolite set enrichment and pathway topology analysis. Pathway enrichment and topology analysis were carried out using a global test and a relative-betweenness centrality measure. Results obtained are depicted in Figure 4A, where the color and the size of each circle indicate its *p*-value and pathway impact value, respectively. The folate biosynthesis pathway was found to be significantly altered (*p* < 0.05). The relative abundance of two metabolites in the folate biosynthesis pathway quantified in plasma samples from GC cases and healthy controls are depicted in Figure 4B.

Discussion

It has been reported that pickled vegetables intake increases the risk of gastric cancer,^{3,6,7,19} but its molecular mechanism in gastric carcinogenesis remains unclear. Metabolomics is

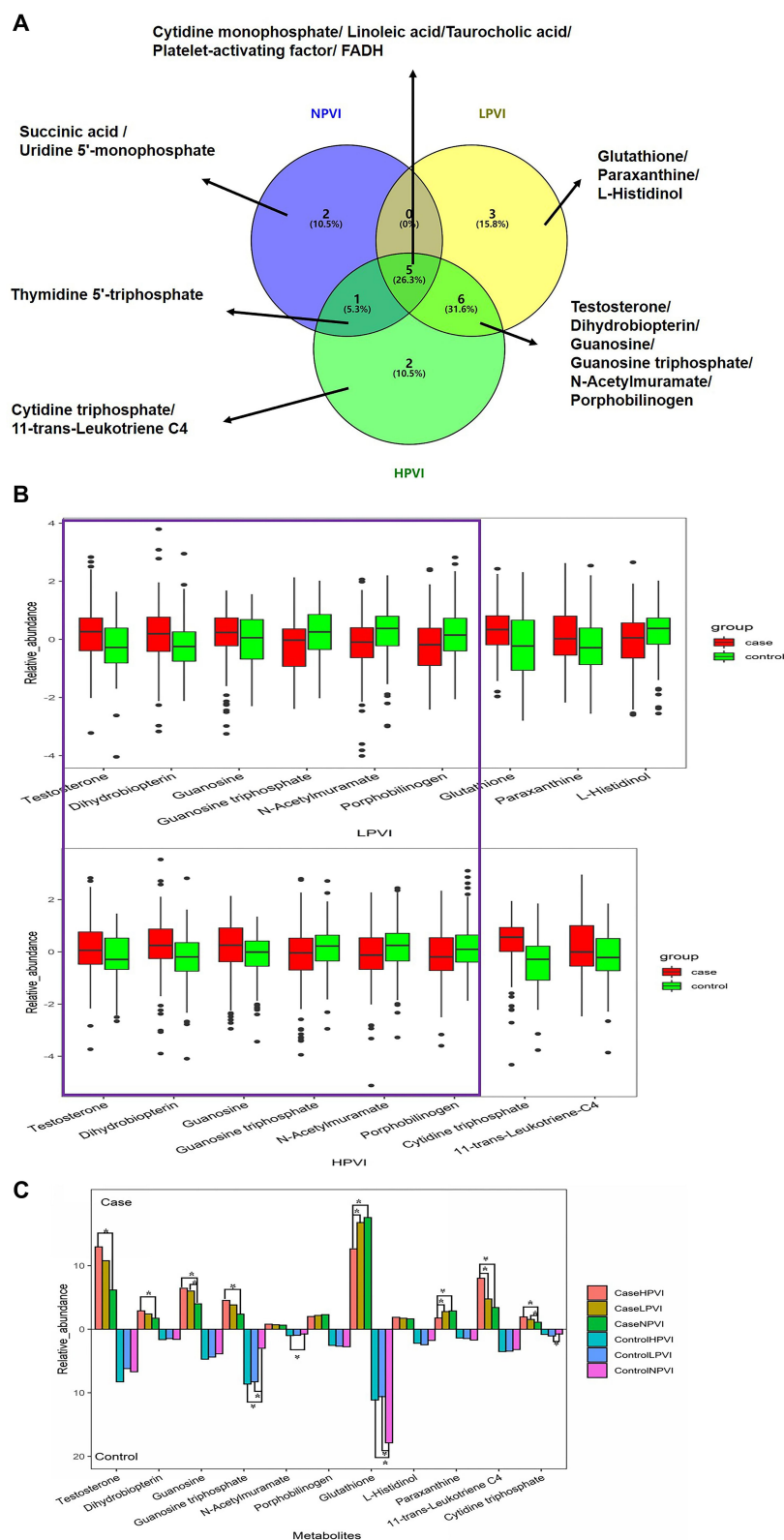


Figure 2 Discriminant metabolites. **(A)** Highly discriminant metabolites (VIP > 1, FDR adjusted t -test p -value < 0.05) between GC cases and healthy controls in the groups NPVI, LPVI, and HPVI. **(B)** Relative abundance of highly discriminant metabolites between GC cases and healthy controls in the groups LPVI and HPVI. **(C)** Relative abundance of highly discriminant metabolites among NPVI, LPVI, and HPVI in the case and control.

Notes: Purple block is the overlapping metabolites of LPVI and HPVI group. * p < 0.05.

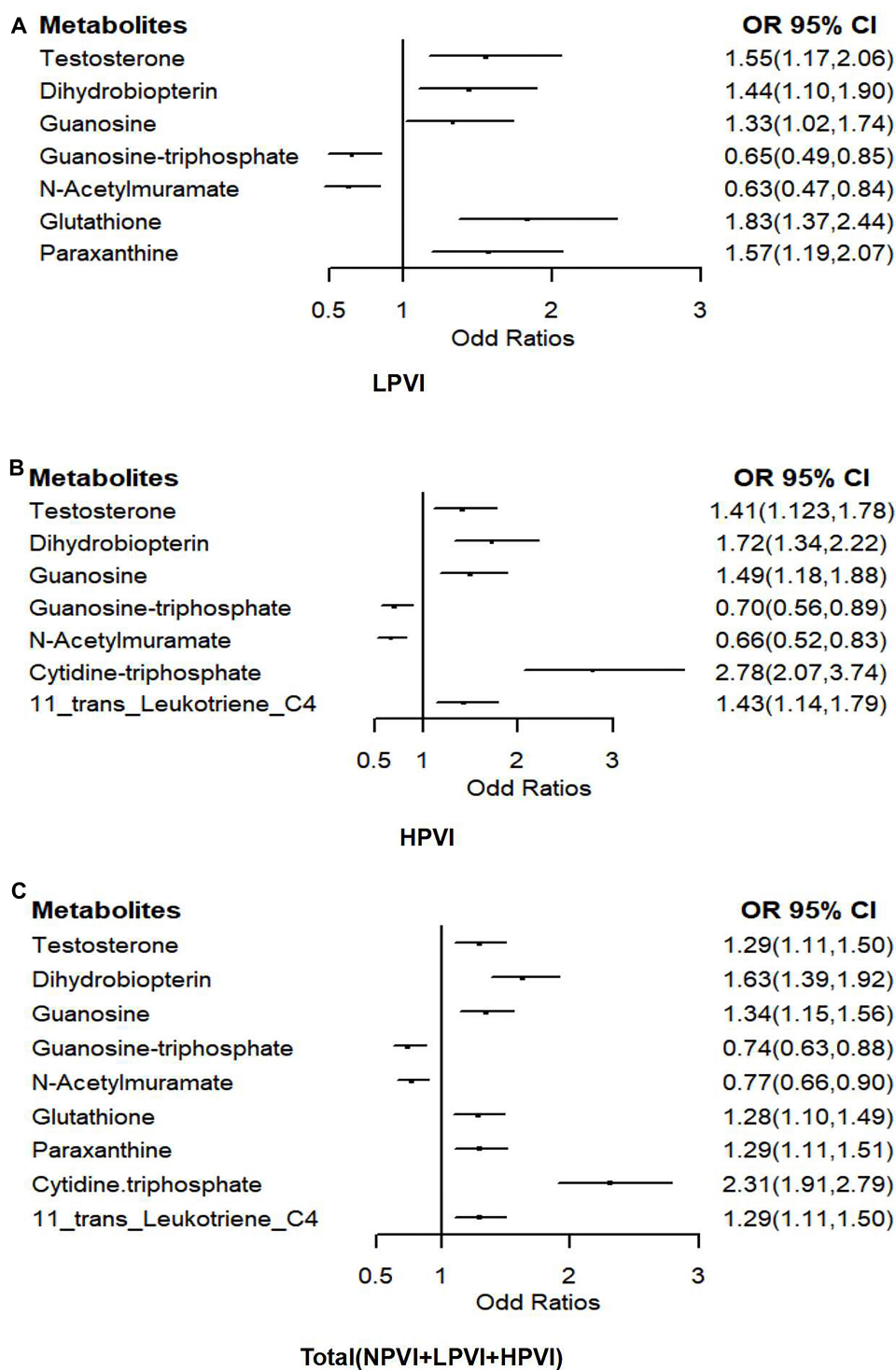


Figure 3 Odds ratios (OR) and 95% confidence intervals (CI) for metabolites related to pickled vegetables intake and the risk of gastric cancer (GC) in the groups LPVI (A), HPVI (B), and total (C).

Notes: OR in LPVI and HPVI is from logistic regression models adjusted for gender; OR in total subjects is adjusted for gender, age, smoking status, and *H. pylori* infection.

now moving beyond biomarkers and towards mechanisms.¹⁶

In this study, we found that nine plasma metabolites were related to pickled vegetables intake, and all of them were associated with the occurrence of gastric cancer, including

seven increased metabolites with increased risks of GC (testosterone, dihydrobiopterin, guanosine, paraxanthine, glutathione, cytidine triphosphate, 11-trans-leukotriene C4); and two decreased metabolites (guanosine triphosphate and

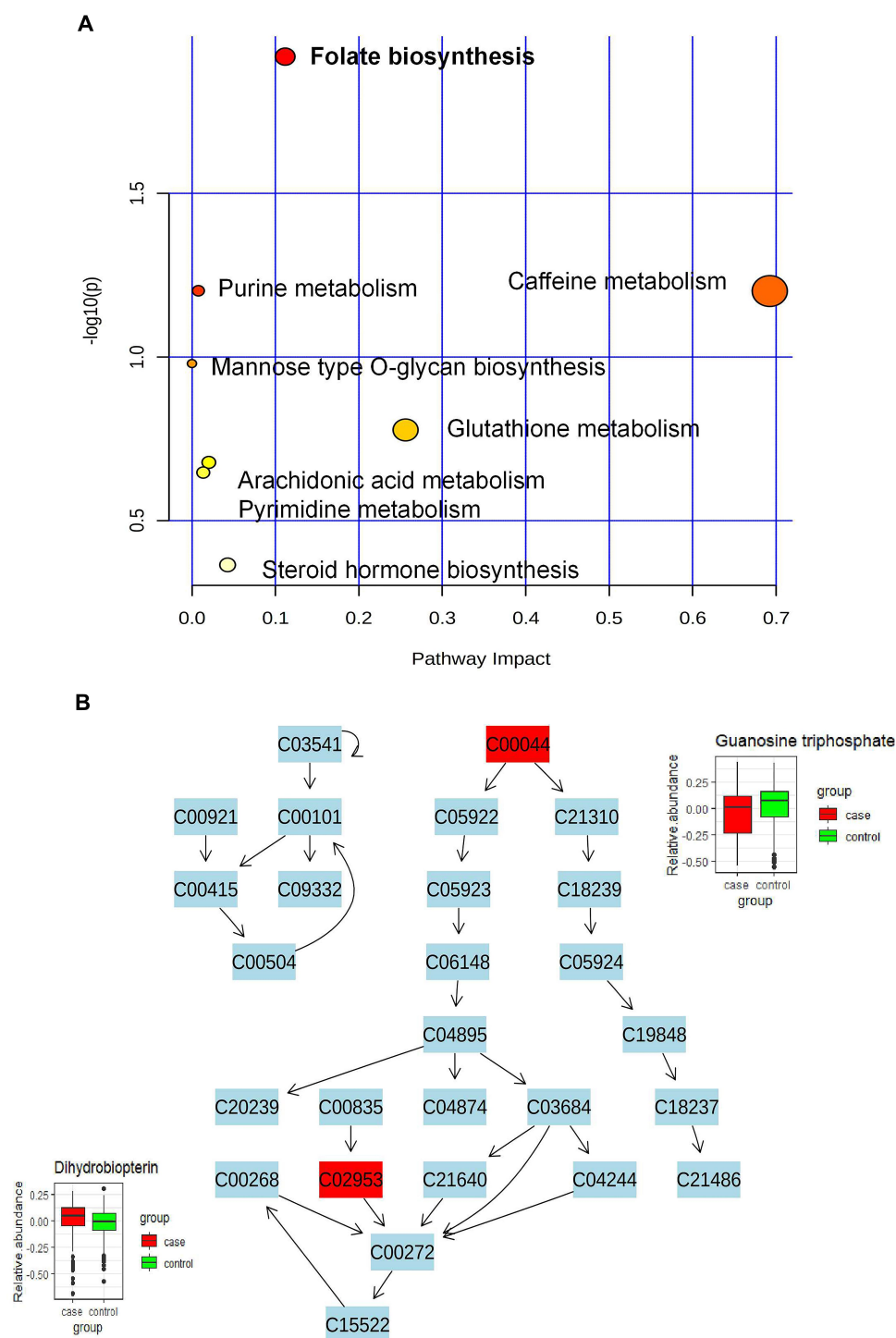


Figure 4 Pathway analysis. **(A)** Pathway analysis of the metabolites related to pickled vegetables intake. **(B)** Overview of differences in the relative abundance of detected metabolites between case and control in the folate biosynthesis pathway.

N-acetylmuramate) with decreased risks of GC. Results from pathway analysis revealed folate biosynthesis pathway was significantly altered ($p < 0.05$).

Folate metabolism is crucial for many biochemical processes, including purine and thymidine monophosphate

(dTMP) biosynthesis, mitochondrial protein translation, and methionine regeneration.²⁰ These biochemical processes in turn support critical cellular functions such as cell proliferation, mitochondrial respiration, and epigenetic regulation.²⁰ Not surprisingly, abnormal folate

metabolism has been causally linked with a myriad of diseases. For example, association between folate status and various forms of cancers has been reported.^{21–24} In this study, we found that the folate biosynthesis pathway was significantly altered in gastric cancer patients. The relative abundance of dihydrobiopterin (BH2) and guanosine triphosphate, the precursor of tetrahydrobiopterin (BH4) of folate biosynthesis, were increased and decreased in gastric cancer patients compared with the healthy controls among pickled vegetables intake groups, respectively. Tetrahydrobiopterin (BH4) is essential for the synthesis of nitric oxide (NO),²⁵ and it is recognized as an essential step in initiating neoplastic transformation.²⁶ A previous study demonstrated that the BH4:BH2 ratio is lower in tumor tissues. As a consequence, nitric oxide synthase activity generates more peroxynitrite and superoxide anion than nitric oxide, resulting in tumor growth and anti-apoptotic signaling.²⁷ In addition, recent evidence suggests that decreasing level of BH4 will weaken the response of CD4 and CD8 T cells, thereby reducing their antitumor activity *in vivo*.²⁸ Tetrahydrobiopterin (BH4) is synthesized by the combined action of three metabolic pathways, namely *de novo* synthesis, recycling, and salvage pathways.²⁹ Guanosine triphosphate (GTP) is the substance of *de novo* synthesis, and reduced GTP will result in reduced BH4 synthesis. BH2 is the metabolic intermediates of BH4 in the salvage pathway catalyzed by dihydrofolate reductase. However, BH4 is easily oxidized to BH2. This explains why the BH2 level of GC cases is higher than that of the controls. Pickled vegetables were made with high concentrations of sodium chloride, which has been reported to induce mucosa and epithelial damage and produce chemical carcinogens—sodium nitrates and nitrites.⁶ We speculate that long-term intake of pickled vegetables might affect the activity of metabolic enzymes in the folate metabolism pathway, which affected BH4 levels and BH4:BH2 ratio and perturbs the folate metabolism pathway, resulting in increased production of peroxynitrite and weakened T cell responses, which ultimately leads to tumor cell proliferation and immune escape. Then tumor cells are in a state of rapid proliferation and differentiation causing nucleotide synthesis and metabolism to be upregulated significantly. For adequate energy generation, guanosine, cytidine triphosphate, and paraxanthine, were increased. Previous research results also showed that hypoxanthine and guanosine were also increased,^{30,31} in agreement with our result.

An increment of glutathione and 11-trans-leukotriene C4 in the GC patients among pickled vegetables intake groups are shown in [Figure 2B](#). Glutathione is the principal intracellular antioxidant buffer against oxidative stress. It participates in many cellular metabolic activities including reactive oxygen species (ROS) removal, DNA and protein syntheses, and signal transduction.³² Cancer cells exhibit a greater ROS level than normal cells in order to meet the enhanced metabolism and proliferation. They have also developed an increased antioxidant defense system to cope with the higher oxidative state.³³ Increased GSH level has been observed in different human cancer cells and is an important contributor to cancer pathology.³⁴ 11-trans-leukotriene C4 is the derivative of leukotriene, which is the major trigger of stress-induced oxidative DNA damage.³⁵ It is well known that pickled vegetables contain exogenous sources of sodium nitrates and nitrites, which can react with amino acids in the stomach and form N-nitro compounds,⁶ resulting in oxidative damage.¹⁶

However, the reduction of N-acetylmuramate (a microbial cell wall component) is catalyzed by N-acetylglucosamine kinase, turning it into N-acetylmuramate 6-phosphate.³⁶ This suggests that plasma metabolism may be affected by intestinal microbes alteration related to pickled vegetables intake. Previous research found that pickled vegetables produced a variety of flora during fermentation.^{37,38} Furthermore, it was reported that N-acetylmuramate was significantly decreased in the context of HLA-B27 expression and functionally modulate spondyloarthritis in a rat model, which is associated with an altered intestinal microbiota and bowel inflammation.³⁹

This study has some limitations. Firstly, data on pickled vegetable intake 10 years ago came from a retrospective survey, and there may be some recall bias. In addition, the detection of metabolites used non-target metabolomics analysis. Although more related metabolites can be found, the structure of some metabolites have not yet been characterized. Therefore, it is necessary to use target metabolomics to further validate specific metabolites. Finally, our metabolites were tested in plasma. Despite the fact that this can provide a starting point for finding links between diseases and metabolic pathways, it may not be the best sample for mechanistic research. Tissue analysis is perhaps more powerful for studying localized and specific responses to stimuli and pathogenesis.¹⁶ Metabolomics analysis using tissue and

animal-based approaches as well as in vitro techniques will be carried out in future research.

Conclusion

In summary, we have reported that nine metabolites and the folate biosynthesis pathway were significantly altered in GC patients among the pickled vegetable intake groups. Our study provides new insights from a metabolic perspective on the relationship between pickled vegetable intake and the occurrence of gastric cancer.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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