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GC/MS-based differential metabolic profiling of human peptic ulcer disease to study *Helicobacter pylori*-induced metabolic perturbations

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Abstract: *Helicobacter pylori* infection has been significantly linked to Peptic Ulcer Disease and Gastric Cancer. Metabolomic fingerprinting may offer a principal way of early diagnosis and to understand the molecular mechanism of *H. pylori*-induced pathogenicity. The rationale of the study is to explore the underlying distinct metabolic mechanisms of *H. pylori*-induced PUD and to identify potential biomarkers for disease diagnosis and associated risks using Gas chromatography/mass spectrometry. GC/MS-based analytical method was used to compare metabolic profiles of healthy controls (N = 20) and peptic ulcer patients (N = 45). Acquired metabolomic data were analyzed by constructing a diagnostic model using principal component analysis and a non-parametric two-tailed paired Wilcoxon analysis to identify disease-specific metabolic biomarkers. A total of 75 low-molecular-weight endogenous metabolites were detected during comparative metabolomic analysis of PUD *vs.* healthy gut tissues, among which 16 metabolites are being proposed to be diagnostic markers of Human PUD. Perturbations related to amino acids, carbohydrates, fatty acids, organic acids, and sterol metabolism were significantly revealed during this differential metabolomic profiling. Results convincingly suggest that metabolic profiles can contribute immensely in early diagnosis of the disease and understanding molecular mechanisms of disease progression for predicting novel drug targets for prophylactic and anaphylactic measures.

Introduction

A peptic ulcer results from an imbalance between damaging forces of gastric acid-pepsin and gastro-duodenal mucosal defense barriers (Chung and Shelat, 2017). Peptic ulcer disease (PUD), being most prevalent in third-world countries, is responsible for 4% of the total mortality among digestive diseases. In addition, as per the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), 14.5 million Americans and about 350,000 new PUD cases are diagnosed with PUD every year (Chai, 2011). Among various endogenous and exogenous stress factors, smoking, nutritional deficiencies, gastric acid, dyspepsia, prolonged intake of non-steroidal anti-inflammatory drug use (NSAIDs), and alcohol are the factors associated with peptic ulcers (Li *et al.*, 2014). In 70–90% of cases, peptic ulcers are

found to be associated with spiral-shaped *Helicobacter pylori* bacteria that reside in the acidic environment of the stomach (Ali, 2013). Interestingly, *H. pylori* infection leads to the development of antral-predominant gastritis that is being linked with gastric hyperchlorydria and duodenal ulcer (DU). Further, it can develop *corpus* gastritis, gastric atrophy, peptic ulcer, and an increased risk of non-cardia gastric cancer (Roesler *et al.*, 2014). Hence, early *H. pylori* eradication is documented with decreased risk of carcinoma in patients with PUD (Take *et al.*, 2005; Wu *et al.*, 2009). At present, the most frequently used technique for early cancer diagnosis is the endoscopy-based pathohistological study of tissue biopsies which is quite complicated (Trivanovic *et al.*, 2019). Hereby, empirical knowledge of disease progression and biomarkers at the molecular level are attractive propositions in order to diagnose and treat PUD patients at an early stage (Song *et al.*, 2012). Thus, metabolomics offers a robust opportunity for understanding various metabolic perturbations and identifying possible biomarkers for early

diagnosis of PUD and further might assist in proposing efficient therapeutics/drug targets for the treatment of the PUD at an early stage before its progression into gastric cancer.

Metabolomics refers to the study of endogenous and exogenous metabolites in biological systems to provide comparative semi-quantitative information about all metabolites in the system ranging from cell cultures to human biological fluids such as urine, saliva, tissue, and blood. Metabolomics is an emerging and potentially powerful tool in discovering biomarkers and identifying perturbed pathways due to disease, which can clarify the mechanism of action (Song et al., 2011; Tan et al., 2016). For metabolomic profiling, gas chromatography/mass spectrometry (GC/MS) has proven to be among the best analytical technique due to its high sensitivity, peak resolution, and reproducibility (Jonsson et al., 2004; Griffin and Shockcor, 2004).

In the present study, we hypothesized that the GC/MS-based inclusive analysis of tissue metabolites symbolizes the markers that can discriminate the PUD group from the normal mucosal group. Our study demonstrated major metabolic perturbations in the antral gastric regions of H. pylori-infected PUD patients that may contribute to the identification of metabolic biomarkers indicating the development of PUD.

Materials and Methods

Ethics statement

Institutional Ethics Committee at Punjabi University, Patiala, has approved the protocol of this study (vide IEC approval no. IEC/05/09/15). As per protocol approved by the review board, written informed consent was taken from all the subjects prior to sample collection. The study using human subjects was performed in accordance with the approved ICMR guidelines and regulations.

Patient recruitment, sample collection and histopathological evaluation

Antral biopsies from peptic ulcer patients (N = 72) and healthy volunteers with normal mucosa (N = 20) were collected from freshly isolated resections using Upper Gastrointestinal Endoscopy (UGIE) carried out by trained professionals at Government Medical College & Hospital, Sector-32, Chandigarh (Punjab, India).

Inclusion criteria: H. pylori-infected PUD patients, as identified in the histopathological evaluation, were enrolled for the present study. Complete medical history of the enrolled patients was recorded as they should not be suffering from any kind of metabolic disorder such as diabetes, hyperthyroidism, or cancer. In addition, healthy subjects were also carefully monitored with no signs of any peptic ulcer/infection and other dietary habits like smoking, drinking alcohol, or stress factor.

Exclusion criteria: Pregnant women, children, immuno-compromised patients, drug abusers (NSAIDs or antibiotics), illiterate and mentally challenged were not considered for the present study.

The tissue biopsies were collected from the margin of the ulcer, cleaned using saline solution, and finally using liquid nitrogen were snapped-frozen within 30 min of collection. The collected biopsies were then investigated for H. pylori infection using hematoxylin and eosin (H&E) stain as stated by Fischer et al. (2008) at the Department of Pathology, Government Medical College & Hospital, Chandigarh. Total cases (normal 20, PUD 45) enrolled for the study included 43 males (66%) and 22 females (34%), ages ranged from 20 to 80 years (patient demographics are given in Suppl. Tab. S1). For further study, biopsies of normal healthy mucosa and H. pylori-infected antral ulcer tissue were considered. Samples were then labeled and stored at −80°C until processing.

Sample preparation for gas chromatography/mass-spectrometry analysis of the gastric tissue specimens

Fifteen milligrams of antral biopsy samples were transferred to 2.0 mL Eppendorf tube and extracted with 500 μL methanol/chloroform solvent in a ratio of 3:1 (v/v) and vortexed for 30 s. The samples were then ultrasonicated (30% amplitude, with pulses 30 s ON, 10 s OFF) for 15 min and vortex-mixed for 30 s. Samples were stored for 10 min at −20°C and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and transferred to a fresh vial and stored at −20°C until analysis. For GC/MS analysis, samples were prepared by adding 100 μL of ethyl acetate (EA) to each tissue extract and vortex-mixed for 1 min. Then, 100 μL of derivatization reagent, consisting of a mixture (3:1:1, v/v/v) of N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), pyridine, and EA, was added, and samples were incubated for 16 h at room temperature. The resulting solution was then vortexed for 1 min, and GC/MS analysis was performed.

GC/MS analysis

Using Perkin Elmer Clarus 500 Gas Chromatography system outfitted with DB-5MS capillary column (25 × 0.2 mm ID, 0.25 μm film thickness) having Perkin Elmer Clarus 500 annual injector analysis was performed. Helium at a constant flow rate of 1.0 mL/min was used as the carrier gas. An aliquot (1 μL) of each of the derivatized solutions was injected in splitless mode. The temperature of the inlet (270°C), transfer line (260°C), ion source (200°C) and quadrupole (150°C) were maintained, respectively. The chromatogram temperature program was run at 80°C (isothermal heating) for 2 min, followed by the sequential rise in oven temperature at the rate of 10 °C/min to 180°C, 5 °C/min to 240°C, 10 °C/min to 260°C and finally maintained at 280°C for 5 min. Data acquisition in MS was achieved in an electron impact mode at 60 eV and scanned for m/z values ranging from 5 to 400 with an acquisition rate of 20 spectra/s with 6 min of solvent delay time (Song et al., 2011).

Statistical analysis

After analysis, each sample was symbolized by a GC/MS total ion current (TIC) chromatogram constituting various peaks. For further study, peaks detected in at least 80% of the samples were considered and analyzed by comparing their respective MS spectra and retention indices with reference data in the Mass Spectral Library of the National Institute of Standards and Technology (NIST) (Wiley Registry,

9th edition, 2008). The mass spectra of each metabolite were vigilantly investigated, and compounds with matching probability >80% were considered for further study. The data in context to ion intensities of respective detected peaks were normalized and then exported to PAST (PAleontological Statistics, Version 3.24) to perform principal component analysis (PCA) with 95% confidence ellipses, where distinctly separate alignment trend was observed in the data. Further, using statistically significant metabolites only (P-value < 0.05) to identify marker metabolites that differentiated PUD and normal groups, a model was proposed. In order to study the statistical significance of the data, a non-parametric two-tailed paired Wilcoxon analysis was performed with SPSS version13.0. P-values of <0.05 were considered statistically significant. False Discovery Rate (FDR)-correction (q values < 0.1) was applied to the P-values to estimate false-positive results according to the standard method of Benjamini and Hochberg. Also, fold change (FC) was calculated by considering a deviation from the median values of 45 PUD specimens relative to that of 20 normal specimens.

Results

For the present study, 20 healthy volunteers were enrolled, keeping in view the exclusion criteria viz a viz no previous medical history related to metabolic disorders or diet and stress-related illness and no intake of NSAIDs, antibiotics, or any kind of drug for at least six months prior to the current investigation. On the other hand, a total of 45 *H. pylori*-associated PUD patients were considered, including 35 males (78%) and 10 females (22%) with a mean age of 46.27 ± 14.8 years. Among the PUD group, the most affected age group was found to be 30–50-year-old (51.1%), and most of them were underweight as per their BMI. However, among 35 infected males, 46% were reported to be non-vegetarian, 43% were regular smokers, and surprisingly 63% were found to be alcoholic; whereas, in the case of females, 80% were having non-vegetarian diets, 20% were smoking, and 30% have been reported to be suffering from psychological stress as mentioned in Suppl. Tab. S1. The study does not provide direct correlations of age, gender, diet, and smoking with PUD development, as the cohort size was very limited in the study.

Histopathology of antral biopsy specimens

The presence of *H. pylori* was not observed in any of the antral biopsies collected from normal healthy volunteers. However, *H. pylori* infection was reported in the gastric mucosa of the peptic ulcer test group by histopathological evaluation, whereas the ulcer biopsies with *H. pylori*-negative evaluation were not considered for further study. Intraepithelial lymphocytes were usually absent in gastric mucosa. However, as evidenced from Fig. 1, breaches in the gastric epithelium were observed in *H. pylori*-infected tissue specimens. Necrotic changes were noticed at the base of the ulcer with no morphological changes at the periphery. In comparison to normal healthy mucosa, *H. pylori*-infected epithelium was reportedly showing signs of dense lymphomononuclear infiltrate migration of mononuclear and polymorphonuclear leukocyte and aggregation of
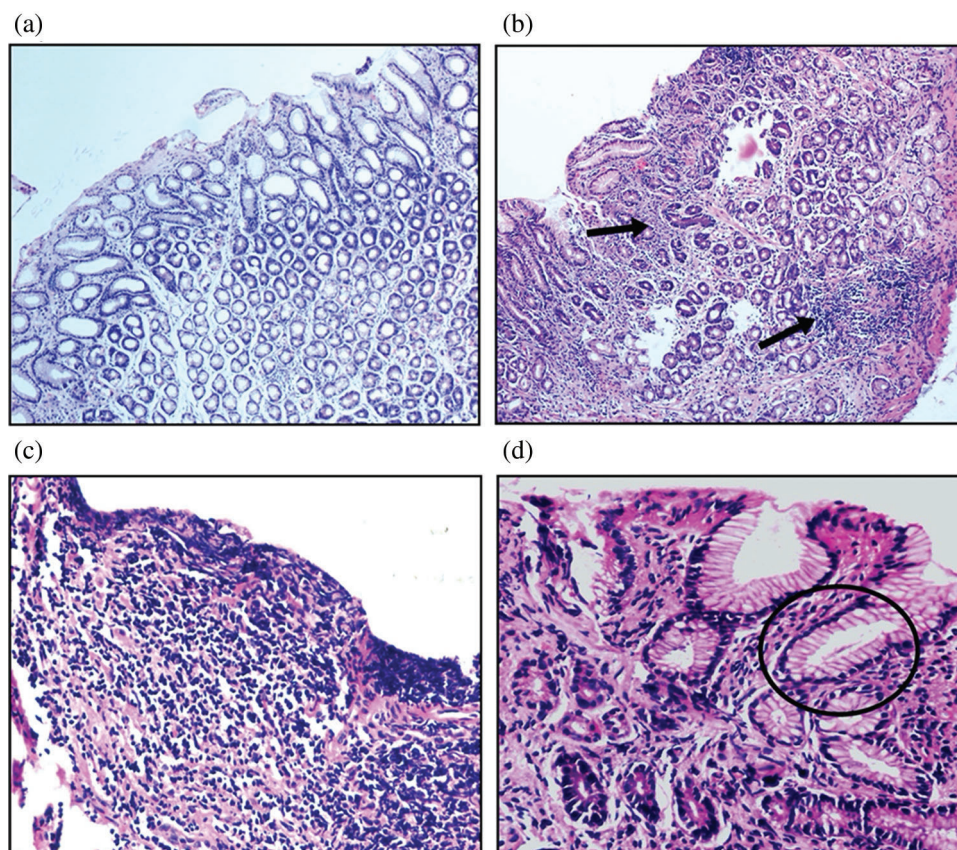
FIGURE 1. Histopathological evaluation of antral gastric biopsies of normal and *H. pylori*-infected PUD patients (H&E-stained), where (a) healthy mucosa in normal patients (100×), (b) mucosal damage due to necrosis of cells, acute inflammation, proliferation with necrotic debris (40×), (c) area of ulceration with the presence of dense lymphomononuclear infiltrate (400×) and (d) showing the presence of microorganisms conforming to the morphology of *H. pylori* within the gastric glands (400×).

lymphocytes which is generally followed by *H. pylori* proliferation in the necrotic debris. The deterioration of gastric epithelium was observed in the *H. pylori*-infected PUD patients that may increase the risk of its progression to gastric carcinoma due to damaging forces of the gastric secretions and the activity of the pathogenic strain if the peptic ulcer remains untreated for a long time.

Metabolomic profiling of healthy and H. pylori-infected PUD tissue specimens

Under optimal GC/MS conditions as mentioned earlier, typical representative total ion chromatograms (TIC) of tissue samples were derived for peptic ulcer patients and the normal controls as presented in Fig. 2. Within one TIC chromatogram, consistently, an average of 90 signals were
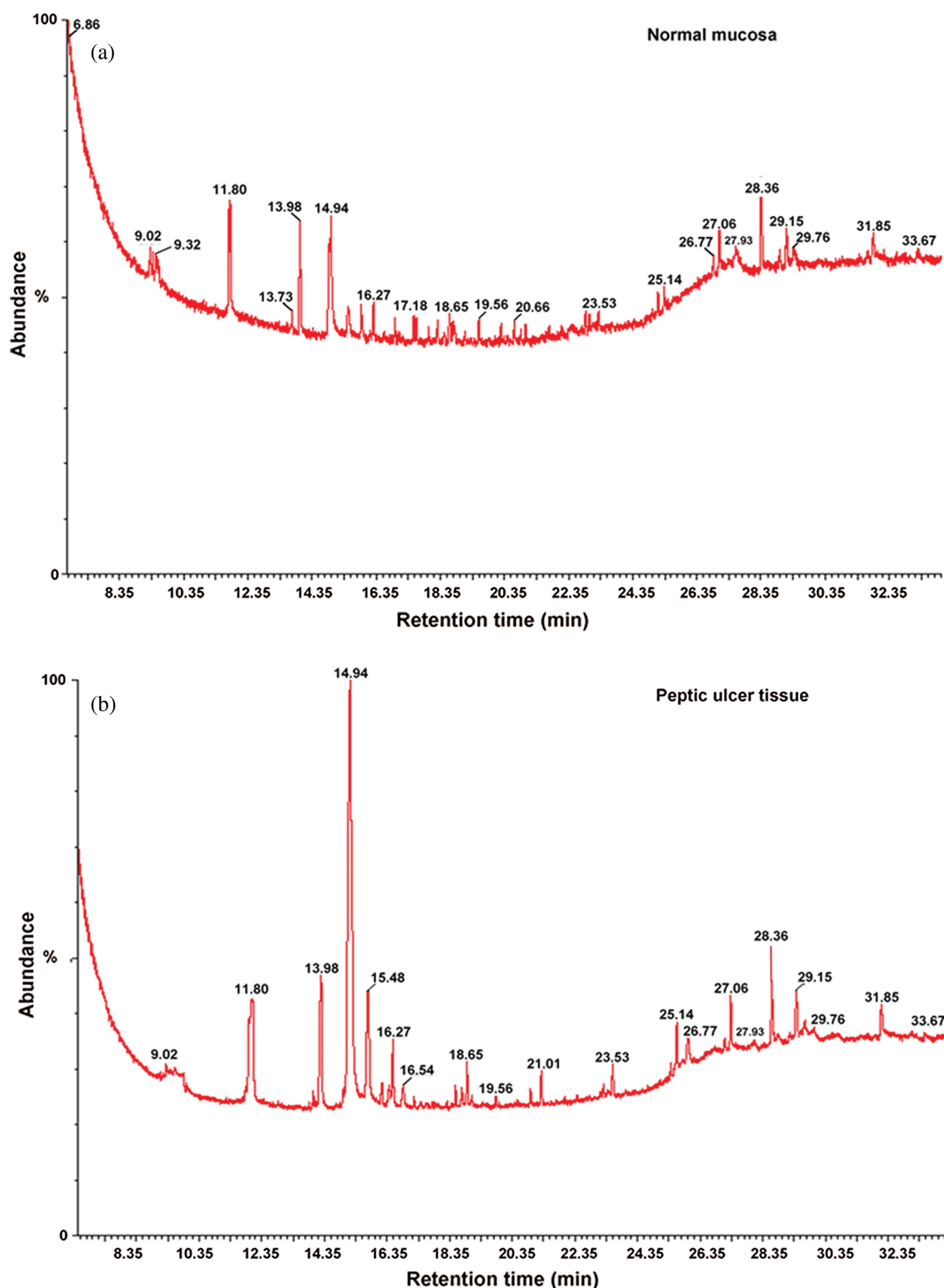

FIGURE 2. Typical GC/MS total ion current (TIC) chromatograms.
(A) Chromatogram of normal mucosa, and (B) chromatogram of peptic ulcer tissue.

sensed, of which some peaks were not taken into consideration due to low abundance and poor spectral quality to be declared as metabolites. In the study, the majority of peaks were identified as low-molecular-weight endogenous compounds based on NIST Mass Spectral Library. As mentioned in Tab. 1, a total of 75 compounds, such as amino acids, carbohydrates, fatty acids, organic acids, and steroids, being involved in various biochemical pathways like amino acid, energy and lipid metabolism were detected in the ulcer and normal mucosal tissues.

Contrastingly, 15 metabolites viz a viz 4-hydroxy-2,6-dimethyl heptane, arachidic acid (C20:0), butyric acid (C4:0), caprylic acid (C8:0), D-mannose, l-(+)-lactic acid, l-arginine, methylmalonic acid, naphthalene, norephedrine, oleanitrile, peroxyergosterol, R-(ar)-tumerone and cysteamine were generated in ulcer tissues but absent in normal mucosal tissue while cholesterol is the most depleted metabolite in ulcer tissues. However, four metabolites, i.e., naphthalene, oleanitrile, peroxyergosterol and R-(ar)-tumerone were not considered for further study as these are main constituents of different food sources and moreover not reported in human gastric metabolic profile yet. Further, the PCA scatter plot, as shown in Fig. 3, satisfactorily confirmed the statistical model to discriminate between the PUD and normal groups when plotted for the obtained.

Analysis of the perturbed metabolites in PUD

To investigate differences in the biomolecular composition of the peptic ulcer and normal mucosal samples, the Wilcoxson test was performed on statistically significant hits ($P < 0.05$) that resulted in the distinct separation of tissues in the PCA plot. Consequently, a total of 45 metabolites were found to have a significant probability ($P < 0.05$) that discriminated metabolic profiles of the two groups as shown in Tab. 1. Interestingly, 29 metabolites were found to be upregulated, while 16 were down-regulated in ulcer tissues. Additionally, l-isoleucine (FC, 13.353), β-hydroxy pyruvic acid (FC, 9.961), stearic acid (FC, 6.042), valeric acid (FC, 3.857), propionic acid (FC, 3.024), and palmitic acid (FC, 0.021) were significantly upregulated in the ulcer tissues, while metabolites like arachidic acid (C20:0), butyric acid (C4:0), caprylic acid (C8:0), l-(+)-lactic acid, l-arginine, methylmalonic acid, norephedrine, and cysteamine were detected only in the PUD tissue specimens; whereas, cholesterol was the most depleted metabolite found in PUD tissue specimens.

Discussion

Metabolic profiling of gastric cancer using various analytical techniques has been reported earlier (Abbassi-Ghadi *et al.,* 2013;

TABLE 1

Differential tissue metabolites of Peptic ulcer tissue and normal mucosa derived from GC/MS chromatograms

No.	RT (min)	Compound	Observed m/z values	Match (%)	FC	*P*-value	*q*-value
		Amino acids, peptides, and analogues					
1	6.13	β-alanine	81.1	99	3.14	0.035	0.061
2	7.49	l-Aspartic acid	70.4	82	−0.096	0.046	0.065
3	8.06	l-Arginine	48.1	99	NA	NA	NA
4	9.32	l-Valine	120.0	97	0.662	**	***
5	10.12	Glycine	67.0	86	−0.111	0.001	0.011
6	11.21	l-Isoleucine	132.1	98	13.353	0.0001	0.002
7	13.35	l-Leucine	73.1	96	0.336	**	***
8	14.05	l-Alanine	90.0	80	−0.613	0.003	0.018
9	15.90	l-Threonine	72.1	80	−0.080	0.04	0.058
10	19.80	l-Proline	116.07	80	−0.059	0.001	0.009
11	21.01	l-Cysteine	168.2	85	0.498	0.001	0.008
12	22.96	l-Glutamine	129.0	80	1.831	0.03	0.054
		Carbohydrates and carbohydrate conjugates					
13	6.35	Arabinose	204.0	83	0.028	0.025	0.046
14	7.64	β-D-Galactopyranoside	217.06	96	1.128	0.024	0.046
15	16.14	Xylonic acid	144.1	94	−0.1	0.02	0.039
16	26.77	Meglumine	41.1	83	−0.907	**	***
17	28.90	D-Mannose	58.2	98	NA	NA	NA
18	31.85	D-Glucose	207.0	82	1.498	0.04	0.065
19	32.09	Arabinopyranose	206.1	80	0.995	0.01	0.034

(Continued)

Table 1 (continued).

No.	RT (min)	Compound	Observed m/z values	Match (%)	FC	P-value	q-value
			Fatty acids and conjugates				
20	13.98	Valeric acid	71.0	88	3.857	0.001	0.007
21	14.77	Dodecanamide	147.0	94	0.068	0.039	0.065
22	15.50	2-Dodecenol	43.1	91	NA	NA	NA
23	16.54	Palmitic acid	78.1	94	0.021	0.02	0.050
24	17.18	1-Hexadecen-3-ol	62.0	93	2.686	0.002	0.013
25	18.65	Nonahexacontanoic acid	47.1	83	1.309	0.003	0.016
26	19.11	Stearaldehyde	48.3	92	−0.059	**	***
27	20.66	Stearic acid	101.2	80	6.042	0.01	0.033
28	22.07	Arachidonic acid	254.1	90	−0.645	**	***
29	23.00	Arachidic acid	68.1	89	NA	NA	NA
30	27.31	Caprylic acid	189.1	96	NA	NA	NA
31	30.60	Propionamide	73.0	80	0.674	0.04	0.063
32	31.13	1-Heptadecanol	62.0	80	0.155	0.003	0.013
			Organic acids and derivatives				
33	6.62	Propionic acid	73.1	84	3.024	0.058	0.077
34	7.29	Methylmalonic acid	68.1	97	NA	NA	NA
35	7.79	Acrylic acid	79.0	80	−0.015	0.01	0.026
36	7.86	β-hydroxy pyruvic acid	132.1	91	9.961	0.0001	0.002
37	10.68	Maleamic acid	152.4	93	0.416	0.01	0.031
38	11.73	Butyric acid	69.1	85	NA	NA	NA
39	13.83	l-(+)-Lactic acid	187.1	99	NA	NA	NA
40	14.59	α-ketoglutaric acid	176.0	90	−0.072	0.004	0.016
41	14.94	Eicosyl acetate	88.0	93	15.922	0.0002	0.003
42	19.56	Hydrocinnamic acid	198.5	86	−0.582	**	***
43	28.36	1,2-benzenedicarboxylic acid	201.4	88	0.047	0.03	0.054
44	29.15	Thioglycolic acid	145.1	88	2.136	0.05	0.068
45	33.67	Glycinamide	69.5	80	−0.343	0.02	0.048
			Others				
46	5.57	Bicarbamamide (Biurea)	41.1	87	4.34	**	***
47	6.86	Phenylephrine	68.0	80	−0.997	0.006	0.022
48	7.17	Phenanthrenol	45.0	80	−0.863	0.003	0.013
49	7.93	4-Hydroxy-2,6-dimethyl heptane	40.1	81	NA	NA	NA
50	7.98	β-mercaptoethylamine (Cysteamine)	167.2	89	NA	NA	NA
51	8.49	3,5-dimethyl-benzaldehyde	76.1	93	9.09	0.01	0.027
52	8.51	2-phenoxyethanol	43.0	88	−0.435	**	***
53	8.79	Butanetriol	63.1	81	1.254	**	***
54	9.02	D-Sphingosine	97.2	90	−0.749	**	***
55	11.79	Oxetane	41.0	81	−0.550	0.0001	0.003
56	11.80	Anthrone	63.0	84	0.842	0.047	0.065
57	12.09	N-Docosane	58.1	96	−0.994	0.02	0.046
58	12.29	Phenol	87.0	84	−0.185	**	***
59	13.53	Phosphate	52.1	80	−0.696	0.005	0.019
60	14.56	R-(ar)-Tumerone	67.1	80	NA	NA	NA
61	15.48	(R)-Pantolactone	77.1	83	5.130	**	***
62	16.27	Dodecane	87.1	94	0.407	0.01	0.030

(Continued)

No.	RT (min)	Compound	Observed m/z values	Match (%)	FC	P-value	q-value
63	18.26	Coumarin	58.0	80	1.503	0.02	0.045
64	18.59	Oxolane	61.1	82	−0.853	0.01	0.028
65	20.14	Naphthalene	127.0	84	NA	NA	NA
66	23.53	Guanosine	69.0	80	0.601	0.02	0.043
67	25.14	Halostachine	49.0	85	−0.223	0.04	0.062
68	25.32	Oleanitrile	67.0	83	NA	NA	NA
69	27.86	Oct-3-ene	78.1	97	0.180	0.035	0.060
70	28.85	Cholesterol	378.1	94	NA	NA	NA
71	29.76	dl-Amphetamine	203.1	82	−0.956	**	***
72	30.08	Norephedrine	143.0	91	NA	NA	NA
73	31.90	1-Decene	71.1	89	−0.871	**	***
74	32.58	Peroxyergosterol	219.1	89	NA	NA	NA
75	33.73	β-phenyl ethylamine	103.1	80	0.248	0.02	0.042

Note: **Insignificant P-value ($P < 0.05$). *** Insignificant q-value ($q < 0.1$).
NA, Not Applicable (FC cannot be calculated because these metabolites are absent in normal gastric metabolic profile except cholesterol, which is absent in PUD group but present in normal one).
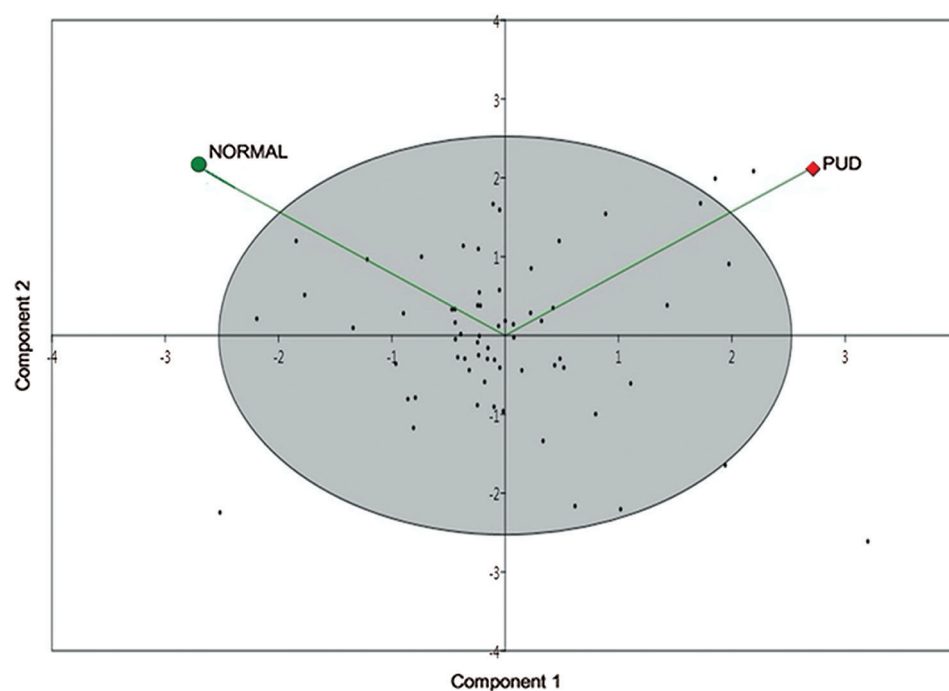

FIGURE 3. Establishment of the PCA model.
The figure illustrates the scatter plot of PCA analysis for tissue metabolomic data. The normal mucosa group tends to cluster to the left region, while the PUD tissue group generally clustered to the right area. The analysis demonstrated satisfactory modeling and achieved a fairly distinct separation between the metabolite profiles of the two groups.

Chan et al., 2014), while none have actually focused on the exploration of the differential gastric metabolic profile of the peptic ulcer in humans. The present study thus aimed at investigating comparative tissue metabolomic fingerprinting of PUD patients vis-a-vis healthy controls. In view of the fact that plenty of variables are interconnected with the metabolomic data, so in order to screen and select differential metabolites, PCA was performed.

From the GC/MS analysis, several marker metabolites related to peptic ulcer disease have been discovered. Several intermediates of "Aerobic Glycolysis" (glucose, β-D-galactopyranoside, l-(+)-lactic and β-hydroxypyruvic acid) were found to be up-regulated in the peptic ulcer tissue,

which is also supported by a previous study on gastric cancer (Chan et al., 2014). Under aerobic conditions, cancer cells prefer aerobic metabolism over the most efficient oxidative phosphorylation for the synthesis of ATP, stated as the 'Warburg Effect' (Alfarouk et al., 2014). Despite the ample availability of oxygen, pyruvate in the gastric tissues is converted into lactate and other metabolites that may particularly benefit the proliferating cancerous cells (vander Heiden et al., 2009). In addition, it has also been reported that lactic acid downregulates the cytotoxic T-cell/NK cell function, thereby inhibiting the differentiation of monocytes to dendritic cells resulting in the immune escape of the cancer cells (Xiao and Zhou, 2017).

Under excess glucose conditions, rapidly proliferating cancer cells tend to be highly glycolytic (Crabtree Effect). Due to disruption of oxidative phosphorylation, they tend to become more sensitive to mitochondrial dysfunctioning (Aguer et al., 2011). In the normal cells, endogenous galactose is phosphorylated to galactose-1-phosphate by the activity of galactokinase (GALK1). Galactose-1-phosphate uridylyltransferase (GALT) further converts galactose-1-phosphate to glucose-1-phosphate that enters glycolysis to yield energy. UDP-galactose is consumed as galactosyl donor for the biosynthesis of glycoproteins and glycolipids, which are crucial for cellular metabolism (Fig. 4). However, in the case of GALT deficiency, a lethal disease called

Galactosemia that is frequently observed in cancer patients, galactose is accumulated in the cells leading to various syndromes like hepatomegaly, bleeding disorder, E. coli sepsis, and death. In addition, excess galactose is also converted into toxic metabolites like galactitol and galactonate. Galactitol further results in the accumulation of hydrogen peroxide and other free radicals, causing oxidative damage to the cells (Lai et al., 2008).

Oxidative stress is involved in natural aging and some human diseases like neurodegeneration, multiple sclerosis, cardiovascular disease, cancer, and rheumatoid arthritis. Elevated levels of reactive oxygen species (ROS), producing free radicals, results in molecular and tissue damage
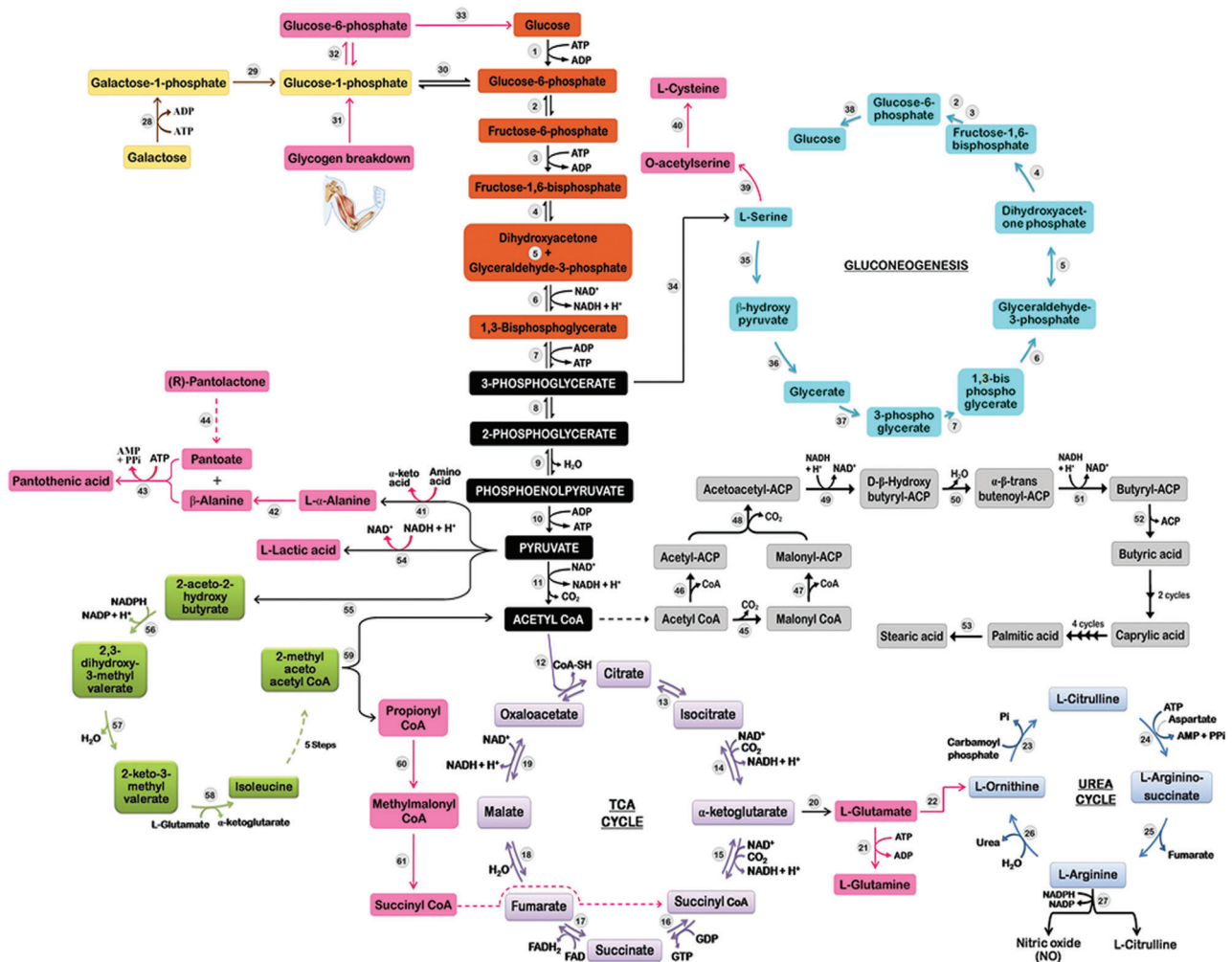
FIGURE 4. Perturbed metabolic pathway analysis in PUD patients using GC/MS; where enzymes catalyzing the respective reactions are as follows: hexokinase (1), phosphoglucokinase (2), phosphofructokinase (3), aldolase (4), triosephosphate isomerase (5), glyceraldehyde-3-phosphate dehydrogenase (6), phosphoglycerate kinase (7), phosphoglycerate mutase (8), enolase (9), pyruvate kinase (10), pyruvate dehydrogenase (11), citrate synthase (12), aconitase (13), isocitrate dehydrogenase (14), α-ketoglutarate dehydrogenase (15), succinyl-CoA synthetase (16), succinate dehydrogenase (17), fumarase (18), malate dehydrogenase (19), glutamate dehydrogenase (20), glutamine synthetase (21), ornithine-oxo acid transaminase (22), ornithine carbamoyl transferase (23), argininosuccinate synthase (24), argininosuccinate lyase (25), arginase (26), NOS (27), galactokinase (28), GALT (29), phosphoglucomutase (30), glycogen phosphorylase (31), phosphoglucomutase (32), glucose-6-phosphatase (33), phosphoglycerate dehydrogenase (34), serine aminotransferase (35), hydroxypyruvate reductase (36), glycerate kinase (37), glucose-6-phosphatase (38), serine acetyl transferase (39), O-acetyl serine sulfhydrolase (40), transaminase (41), alanine-2,3-amino mutase (42), pantothenate synthetase (43), unknown (44), ACCase (45), acetyl-CoA-ACP transacylase (46), malonyl-CoA-ACP transacylase (47), β-ketoacyl-ACP synthase (48), β-ketoacyl-ACP reductase (49), β-hydroxyacyl-ACP dehydrase (50), enoyl-ACP reductase (51), thioesterase (52), elongase (53), lactic dehydrogenase (54), 2-aceto-2-hydroxy butyrate synthase (55), acetohydroxyacid isomeroreductase (56), dihydroxyacid dehydratase (57), isoleucine transaminase (58), 2-methyl acetoacetyl CoA thiolase (59), propionic carboxylase (60), and methylmalonic acid mutase (61).

triggering a chain of destruction to promote oncogenic transformation; thus, free radicals have proven to be a useful index for depicting biological markers *in vivo* (Katerji *et al.*, 2019). In the present study, several oxidative stress markers viz. β-hydroxy pyruvic acid, 4-hydroxy-2,6-dimethyl heptane, β-mercaptoethylamine, and 3,5-dimethyl benzaldehyde have been detected in PUD tissue which indicates the possibility of oxidative DNA damage and hormonal dysbiosis in the diseased patients (Tab. 2). Earlier also, it was reported that 3,5-dimethyl benzaldehyde, obtained from lipid peroxidation, synergizes with DNA damage to induce accelerated stress. Its endogenous routes involve phenyl acetaldehyde through β-oxidation or cinnamic acid through β-oxidation (Flor and Kron, 2016).

Elevated amino acids are the major contributing factors in cancer cells; specifically, many cancer cell lines require l-glutamine, a well-known nutrient for their survival even under metabolic stress (Choi and Park, 2018). Therefore, following a general trend, the level of amino acid biosynthesis is upregulated in cancerous tissues as reported earlier in esophagogastric cancer (Abbassi-Ghadi *et al.*, 2013). A similar disturbance in the amino acid metabolism was also observed in the present study as levels of l-glutamine (FC, 1.831), l-isoleucine (FC, 13.353), and β-alanine (FC, 3.14) were significantly upregulated in the *H. pylori*-infected gastric tissue. Amino acids have proved to be an alternative source of energy generated through anaplerotic reactions. Glutamine, a prime example of anaplerotic reaction, enters the TCA cycle by its conversion into glutamate and α-ketoglutarate (Chan *et al.*, 2014). But in the present study, disruption of the TCA cycle was observed, and subsequent levels of α-ketoglutarate declined in ulcer tissues, which indicate glutamate bioconversion to arginine (via urea cycle) which is detected only in the PUD (Fig. 4). The gluconeogenetic β-alanine (FC, 3.14) and l-glutamine (FC, 1.831) were found to be linked previously with hepatocellular carcinoma (Brosnan, 2003; Xue *et al.*, 2008). The endogenous levels of amino acids cysteine, l-threonine, l-alanine, l-glycine, and l-aspartate showed depletion due to disruption in metabolic changes associated with gastric dysbiosis were previously linked with the etiology of gastric cancer by Wu *et al.* (2010). l-arginine is exclusively reported as an amino acid biomarker of PUD

that represents the progression of the disease as observed earlier in arginine-dependent tumors, i.e., hepatocellular carcinoma, lymphomas, melanoma, sarcomas, and urological cancers (Phillips *et al.*, 2013). Further, branched-chain amino acids (l-isoleucine, l-leucine and l-valine) are reported to be essential for tumor proliferation as they are incorporated directly into proteins or being metabolized. The amino group of branched-chain amino acids is being transferred to α-ketoglutarate in presence of branched-chain amino acid transferase in order to produce glutamate and a branched-chain α-ketoacid that eventually enters the TCA cycle and thus is suggested as a prognostic cancer indicator (Selwan and Edinger, 2017). He *et al.* (2012) stated that perturbations in glutamine and arginine metabolic pathways may contribute to the pathomechanisms and memory deficits associated with Schizophrenia.

Glutamine basically tends to control the master regulator of protein translation mTORC1 required for anabolic growth of cells. In addition, it acts as a nitrogen donor for various metabolic enzymes and *de novo* synthesis of purines and pyrimidines required for proliferating cancer cells (DeBerardinis and Cheng, 2010). l-arginine is required by the proliferating cancer cells for protein biosynthesis, growth and survival. Under the restricted exogenous l-arginine supply, cancer cells may suffer from increased autophagy and apoptosis, and thus, cancer cells are bound to produce an ample amount of l-arginine (Szlosarek, 2014). Additionally, l-arginine is also a precursor of nitric oxide (NO), which is essential for maintaining healthy endothelium. Interestingly, l-arginine can also be catabolized to urea which inhibits the activity of nitric oxide synthase (NOS). Prior studies have demonstrated that NOS inhibition is a risk factor for cardiovascular mortality, diabetes, hypertension, and renal dysfunction (Finkelman *et al.*, 2017).

The homeostasis of amino acids has been regulated by two protein kinases, namely mechanistic target of rapamycin complex 1 (mTORC1) and general control non-derepressable 2 (GCN2), required for cell growth and proliferation in response to numerous growth and stress signals. Increased endogenous levels of l-arginine and l-cysteine significantly activate mTORC1 (sensor of arginine and leucine) and GCN2 kinases. In response to amino acid-uncharged tRNA, kinases result in inhibition of protein

TABLE 2

List of peptic ulcer disease biomarkers reported

PUD Biomarkers			
Amino Acids	l-Isoleucine l-Arginine l-Glutamine β-alanine	Organic Acids	Butyric acid l-Lactic acid Methylmalonic acid Propionic acid β-hydroxypyruvic acid
Fatty Acids	Stearic acid (C18:0) Valeric acid (C5:0) Arachidic acid (C20:0) Caprylic acid (C8:0)	Others	β-mercaptoethylamine 4-hydroxy-2,6-dimethyl heptane 3,5-dimethyl benzaldehyde

translation that further induce the elevation of the cellular amino acid pool (Bar-Peled and Sabatini, 2014; Vučetić et al., 2017). Consequently, glutathione (GSH), linked with increased endogenous levels of l-cysteine, imparts a significant role in cell differentiation, proliferation, and progression to various diseases, including cancer (Traverso et al., 2013).

Organic acids such as l-(+)-lactic acid, methylmalonic acid and butyric acid (fatty acid intermediate) emerged as important biomarkers of PUD. Accumulation of lactic acid and disruption of TCA cycle as reported earlier in cases of colorectal, gastric, liver, and invasive ovarian cancers by Tan et al. (2016). Previous studies have demonstrated that elevated levels of glycolytic flux and lactate excretion are associated with malignant transformation; however, lactate accumulation alone is sufficient to enhance the degree of tumor malignancy and impairment of mitochondrial oxidative metabolism (Redjems-Bennani et al., 1998; Walenta and Mueller-Klieser, 2004). Earlier, Frye et al. (2016) reported that elevated endogenous levels of propionic acid (studied in Autism Spectrum Disorders in children) were metabolized in multiple reactions to succinyl-CoA that inhibited the activity of first (citrate synthase) and fourth (α-ketoglutarate dehydrogenase) enzymes, thus blocking TCA cycle that results in decreased production of NADH.

Another prominent observation recorded in the present study was the perturbation of fatty acid metabolism in ulcer tissues. Metabolomic fingerprinting revealed significant enhancement in the levels of saturated fatty acids (arachidic acid, caprylic acid, nonahexacontanoic acid, stearic acid and valeric acid) significantly increases in PUD tissues; while the reduction in the endogenous level of unsaturated fatty acid (arachidonic acid) was reported as previously observed in case of lung cancer and non-Hodgkin lymphoma (Song et al., 2011; Song et al., 2012). According to the scientific evidence, cancer cells show dysfunction of the β-oxidation pathway of fatty acid biosynthesis and cell membrane synthesis (Chan et al., 2014). Stearic acid (FC, 6.042) and valeric acid (FC, 3.857) were the most elevated saturated fatty acids, while arachidonic acid (FC, −0.645) was the most depleted one. Li et al. (2014) reported the role of stearic acid (also known as Octadecanoic acid) in fatty acid metabolism disorder and rehabilitation of peptic ulcers. Stearic acid promotes ulcer formation by increasing the inflammatory response and mitochondrial dysfunction.

Elevated endogenous levels of methylmalonic acid (MMA) observed in PUD tissue specimens might be because of its inability to convert into succinyl CoA signifying vitamin B12 deficiency (Vashi et al., 2016). Schloss et al. (2013) stated that malabsorption, gastrointestinal surgeries, and intake of medications (proton pump inhibitors, etc.) precede cancer patients susceptible to vitamin B12 deficiency. In PUD, gastric mucosa is not intact, thus unable to produce intrinsic factors leading to vitamin B12 malabsorption. Hence, vitamin B12 was marked as a potential biomarker for atrophic gastritis that increases the risk of gastric adenocarcinoma (Miranti et al., 2017). Further, accumulation of MMA may also result in renal failure, thyroid disease, implicated in the pathogenesis of neuronal damage and Alzheimer's disease

(Bjørke Monsen and Ueland, 2003; Kolker et al., 2000; Serot et al., 2005). While, butyric acid has proven to be an indicator for compromised gut wall integrity, as observed in AIDS subjects (Stein et al., 1997). Short-chain fatty acids such as acetic, butyric, and propanoic acids cause a decrease in the functioning of the mucosal barrier, which may contribute to increased pathogenesis of gastric ulcers through multiple mechanisms such as alternate energy reservoir, acidification of cellular content, impairment of Na-transport channels, and cellular swelling as observed in animal model studies (Nadeau et al., 2003; Załęski et al., 2013). An interesting finding is the depletion of cholesterol in the case of peptic ulcer tissues. The decline in cholesterol synthesis (linked with genes present in chromosome 5) might be due to translational competition, which increases the concentration of adenocarcinoma-associated biomarkers leading to the progression of adenoma to gastric cancer (Song et al., 2011). (R)-Pantolactone is a precursor of pantothenic acid, needed to synthesize coenzyme A and acyl carrier protein (ACP) portion of fatty acid synthetase (Rawalpally, 2001). It is therefore hypothesized that altered levels of coenzyme A and ACP have major implications on fatty acid biosynthesis and the progression of PUD. So, pantothenic acid (vitamin B5) deficiency may be caused in the animals (symptoms common with PUD).

Cysteamine (also known as β-mercaptoethylamine) is a biological compound produced in the gastrointestinal tract and hypothalamus of all animals acting upon the somatotrophic axis. It helps in improving nutrient digestion and absorption by enhancing portal drained visceral blood flow and net portal absorption. Further, cysteamine also downregulates the secretion of gastroenteropancreatic plasma and an inhibitory hormone named somatostatin (Barnett and Hegarty, 2016). In the gastrointestinal tract, somatostatin is present in abundance that helps in the suppression of gastric acid secretion. Altered expression of somatostatin results in increased levels of gastric acid secretion leading to various gastrointestinal diseases/dysbiosis (Minalyan et al., 2017). Also, cysteamine emerged as an important oxidative biomarker of PUD, which has been previously linked with the etiology of Ehlers-Danlos syndrome, severe skin rashes, duodenal ulcers, or bleeding in the intestine (Weintraub, 2015). It also tends to concentrate in the duodenum and generate reactive oxygen species (ROS), resulting in the progression of the ulcer by decreasing the defense activity of superoxide dismutase (SOD), as stated by Elberry (2013). Contrastingly, Borrell-Pagès et al. (2006) reported cysteamine to be a part of treatment for Huntington's disease (HD), as it elevates the level of heat shock DnaJ-containing protein 1b (HSJ1b), which is present in low concentration in HD patients. Norephedrine, an α, β-adrenergic receptor agonist, enhances the secretion of nor-epinephrine hormone that further elevates blood pressure, heart rate, breakdown of galactose to release glucose, inhibits voiding of bladder and gastrointestinal motility or bowel movements (Deedwania, 2015; Konturek et al., 2004; Mareev and Cleland, 2015). As these conditions are frequently reported in PUD patients, thus occurrence of endogenous levels of cysteamine and norephedrine is pointing towards hormonal dysbiosis in PUD patients.

Conclusion

In summary, by using a combinational approach involving highly sensitive analytical techniques and multivariate and univariate statistical tests, significant metabolic shifts and perturbations of amino acids, carbohydrate, fatty acids, organic acids, and sterols were identified in the case of peptic ulcer tissues. The results obtained in the study revealed the potential of the MS-based robust metabolic profiling approach for early diagnosis of PUD and contributed substantial evidence to understand pathophysiological responses during the development of PUD in humans. However, the study could not provide a direct correlation of age, gender, diet, and smoking with the development of PUD, as the cohort size was very limited.

Future Perspective

The results obtained in the study could be utilized to develop serosurvey kits for early diagnosis of PUD using marker metabolites excreted in blood or urine of the *H. pylori*-infected PUD patients. However, a detailed evaluation of the abundance and occurrence of these marker metabolites in blood and urine samples needs a thorough investigation using PUD subjects. In addition, perturbed metabolites detected in the PUD group could lay the foundation for finding novel therapeutic targets and discovering new multi-target drugs. It is hereby proposed that enzymatic dysbiosis is also linked with PUD and differential expression of several candidate genes expressing adenylate kinase, AMP kinase, pyruvate kinase, lactate dehydrogenase, GALT, GCN2 kinase, GSH, branched-chain aminotransferases, SOD, ROS, coenzyme A synthetase, and acyl carrier protein could be explored for understanding a detailed mechanism of development of *H. pylori*-induced PUD and its progression to gastric carcinoma, if untreated.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Authors' Contribution: Research work, results interpretation and manuscript preparation was done by Gaganjot Gupta; research idea and guidance by Dr. Baljinder Kaur; endoscopy and biopsy sectioning were performed by Dr. Deepak Bansal and Dr. Atul Sachdev; Anshula Sharma, Ajaz Ahmad, Tawseef Ahmad and Hamed A. El-Serehy helped in manuscript preparation.

Ethics Approval: All procedures performed in the study were in accordance with the ethical standards of the Institutional Ethics Committee and as per Indian Council of Medical Research (ICMR), New Delhi (India) guidelines and regulations with approval No. IEC/05/09/15 on 10th September, 2015.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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TABLE S1

Patient demographics for cases enrolled for study with informed consent

Group	Total Patients enrolled	Cases under study	Gender (Male/Female)	Age (Years)		BMI		Diet		Smoking	Alcohol	Psychological
				Median	IQR	Median	IQR	V	NV	Consumption		stress
Normal	20	20 (HP -ve)	Male (N = 8)	36	21–56	24.75	17.2–28.3	7	1	0	0	0
			Female (N = 12)	45	28–70	19.35	17.9–29.5	10	2	0	0	0
PUD	72	45 (HP +ve)	Male (N = 35)	39	20–70	20.3	13.8–29.8	19	16	15	22	4
			Female (N = 10)	42	28–70	20.9	17.2–28.3	2	0	2	0	3

Note: *BMI = Body Mass Index; V = Vegetarian; NV = Non-vegetarian; IQR = Interquartile range.