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# Metabolomic Profiling of Plasma Bile Acids in Resectable Gastric Cancer

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**Abbreviations:**
ACN: Acetonitrile;
ApCA: Allo-petracholic acid;
AUC: Area under the curve;
BA: Bile acid / bile acids;
CA: Cholic acid;
CA19-9: Carbohydrate antigen 19-9;
CA72-4: Carbohydrate antigen 72-4;
CDCA: Chenodeoxycholic acid;
CEA: Carcinoembryonic antigen;
CRP: C-reactive protein;
CTR: Control;
DCA: Deoxycholic acid;
DHLCA: Dehydrolithocholic acid;

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## Rezumat

***Profilarea metabolomică a acizilor biliari plasmatici în cancerul gastric rezecabil***

*Introducere:* Cancerul gastric (CG) se caracterizează prin diagnosticarea în stadii avansate şi prin lipsa unor biomarkeri plasmatici neinvazivi cu sensibilitate şi specificitate de diagnostic ridicată. Acest studiu îşi propune să investigheze profilul acizilor biliari (AB) plasmatici pentru a îmbunătăţi înţelegerea metabolismului CG şi pentru a identifica potenţiale instrumente diagnostice şi prognostice.
*Metode:* În cadrul studiului prospectiv s-a folosit un design tip caz-control şi au fost recrutaţi 62 de pacienţi cu CG în stadiile I–III şi 70 de martori adecvaţi cu caracteristicile de vârstă şi de mediu asemănătoare cu cel al lotului oncologic. Utilizând cromatografia lichidă cuplată cu spectrometrie de masă în tandem (LC-MS/MS), au fost măsurate concentraţiile a 48 de metaboliţi plasmatici. Analiza statistică a inclus teste univariate, analiza componentelor principale şi analiza discriminantă liniară (LDA).
*Rezultate:* Pacienţii cu CG au prezentat un raport CA/CDCA semnificativ mai scăzut şi modificări ale acizilor biliari secundari şi conjugaţi, inclusiv TLCA, GLCA, TDCA, GDCA şi GUDCA, sugerând implicarea axei intestin–ficat–microbiom. Capacitatea de diferenţiere între grupuri a fost moderată (AUC = 0,731). În plus, nivelurile AB au fost corelate negativ cu stadiul tumoral, dimensiunea tumorii şi markerii inflamatori sistemici (CRP, mGPS), totodată fiind corelate pozitiv cu markerii nutriţionali şi hematologici, precum albumina şi hemoglobina.
*Concluzii:* Cancerul gastric este asociat cu un profil distinct al acizilor biliari circulanţi, care reflectă nu doar remodelarea metabolică asociată tumorii, ci şi inflamaţia sistemică, statusul nutriţional şi povara bolii. Raportul CA/CDCA redus şi modificările acizilor biliari secundari şi conjugaţi susţin implicarea axei intestin - ficat - microbiom în carcinogeneza cancerului gastric. Deşi profilarea acizilor biliari a

FXR: Farnesoid X receptor;
GC: Gastric cancer;
GCA: Glycocholic acid;
GCDCA: Glycochenodeoxycholic acid;
GDCA: Glycodeoxycholic acid;
GLCA: Glycolithocholic acid;
GUDCA: Glycoursodeoxycholic acid;
H. pylori: Helicobacter pylori;
IDCA: Isodeoxycholic acid;
ILCA: Isolithocholic acid;
LCA: Lithocholic acid;
LC: Liquid chromatography;
LC-MS/MS: Liquid chromatography-tandem mass spectrometry;
LDA: Linear discriminant analysis;
mGPS: Modified Glasgow Prognostic Score;
MDCA: Murideoxycholic acid;
MeOH: Methanol;
MS: Mass spectrometry;
$N_2$: Nitrogen;
NYHA: New York Heart Association;
PCA: Principal component analysis;
ROC: Receiver operating characteristic;
S-Conj: Secondary conjugated bile acids;
TCA: Taurocholic acid;
TCDCA: Taurochenodeoxycholic acid;
TDCA: Taurodeoxycholic acid;
THDCA: Taurohyodeoxycholic acid;
TLCA: Taurolithocholic acid;
TNM: Tumor, Node, Metastasis;
TUDCA: Tauroursodeoxycholic acid;
UDCA: Ursodeoxycholic acid;
WHO: World Health Organization.

demonstrat individual o performanță diagnostică moderată, integrarea acesteia cu markerii tumorali convenționali, indicii inflamatori şi parametrii clinico-patologici ar putea îmbunătăți panourile multimodale de biomarkeri pentru stratificarea non-invazivă a pacienților, evaluarea bolii şi analiza prognostică viitoare.

**Cuvinte cheie:** cancer gastric, acizi biliari, metabolomică, LC-MS/MS, axa intestin - ficat - microbiom

## Abstract

*Background:* Gastric cancer (GC) is characterized by late-stage diagnosis and a lack of reliable non-invasive biomarkers. This study aims to investigate the plasma bile acid (BA) profile to enhance the understanding of GC metabolism and identify potential diagnostic and prognostic tools.

*Methods:* In a case-control design, 62 GC patients (stages I–III) and 70 matched controls were recruited. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), the concentrations of 48 metabolites in plasma were measured. Statistical analysis included univariate tests, principal component analysis, and linear discriminant analysis (LDA).

*Results:* GC patients showed a significantly lower CA/CDCA ratio and alterations in secondary and conjugated bile acids, including TLCA, GLCA, TDCA, GDCA, and GUDCA, suggesting involvement of the gut–liver–microbiome axis. The ability to distinguish between groups was moderate (AUC = 0.731). Furthermore, BA levels were negatively correlated with tumor stage, tumor size, and systemic inflammatory markers (CRP, mGPS), while they were positively correlated with nutritional and hematological markers such as albumin and hemoglobin.

*Conclusions:* Gastric cancer is associated with a distinct circulating BA profile that reflects not only tumor-related metabolic remodeling, but also systemic inflammation, nutritional status, and disease burden. The reduced CA/CDCA ratio and alterations in secondary and conjugated bile acids support the involvement of the gut-liver-microbiome axis in GC biology. Although BA profiling alone showed moderate diagnostic performance, its integration with conventional tumor markers, inflammatory indices, and clinico-pathological parameters may improve multimodal biomarker panels for non-invasive patient stratification, disease assessment, and future prognostic evaluation.

**Keywords:** gastric cancer, bile acids, metabolomics, LC-MS/MS, gut-liver-microbiome axis

## Introduction

Gastric cancer (GC) is one of the most aggressive malignancies worldwide, ranking fifth in incidence and general cancer-related mortality (1). Despite the advances in surgical techniques, chemotherapy protocols and personalized oncological therapies, prognosis for the affected individuals remains poor. In advanced stages, 5-year survival rate is less than 30% (2), largely due to late-stage diagnosis because of the non-specific symptoms, high recurrence rates, and restricted availability of reliable non-invasive biomarkers for early detection and prognosis (3). Therefore, there is an urgent demand for better understanding GC pathogenesis and molecular adaptations of the cancer cells, in order to develop innovative diagnostic tools that can detect GC in early stages, monitor disease progression and are able to predict treatment response.

The most widely used histological system for gastric cancer is the Lauren classification, introduced in 1965, which divides gastric adenocarcinomas into 3 major types: intestinal, diffuse and indeterminate type (4,5). To enhance and elaborate Lauren's contribution to gastric cancer, the World Health Organization (WHO) developed a more detailed histopathological

classification system, which was most recently revised in 2010. The WHO classification recognizes four main histological subtypes of gastric adenocarcinoma: tubular, papillary, mucinous, and poorly cohesive carcinoma, which includes signet-ring cell carcinoma. Each subtype reflects distinct morphologic, clinical, and sometimes molecular characteristics, many of which have unique clinical behaviors and may require specialized therapeutic approaches (6).

Present diagnostic methods for gastric cancer, including endoscopy and histopathological examination, are accurate but invasive, expensive and not suitable for large-scale population screening (7). Conventional serologic markers such as carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and carbohydrate antigen 72-4 (CA72-4) are widely used, but do not possess adequate sensitivity and specificity for early-stage GC detection (8).

Nowadays, bile acids are considered relevant in gastric carcinogenesis because they can contribute to gastric mucosal injury, inflammation, epithelial remodeling, and disruption of mucosal homeostasis, particularly in the context of duodeno-gastric bile reflux (9,10). Recent evidence also supports a mechanistic link between bile acid exposure and gastric intestinal metaplasia, a recognized precancerous lesion of gastric cancer; specifically, bile acid–related signaling has been associated with activation of epithelial reprogramming pathways involving CDX2, MUC2, GATA4, and NF-κB (9,11). Also, the bile acid–gut microbiota axis may be important in creating precancerous gastric lesions, because bile acids and gut bacteria interact bidirectionally, influencing bile acid transformation, mucosal inflammation, immune regulation, and metabolic homeostasis (10,12,13).

Feng et al. conducted a study in 2025 combining plasma metabolomics with 16S rDNA sequencing in advanced gastric cancer, which reported significant differences in both plasma metabolites and fecal microbial communities and showed correlations between intestinal microbiota and plasma metabolites, supporting the concept that microbiota–metabolite interactions may contribute to gastric cancer biology (14). Therefore, bile acid dysregulation should be interpreted not as an isolated metabolic alteration, but as part of a broader network of events involving bile reflux, mucosal inflammation, epithelial metaplasia, microbiota changes, and cancer-related metabolic reprogramming (9,10,12,14).

The comprehensive analysis of metabolites in biological samples, such as plasma and tissue, provides an effective tool to elucidate cancer-specific metabolic alterations and discover novel biomarkers. Compared to conventional genomic and proteomic methods, it has a number of benefits, such as the capacity to identify early metabolic disturbances linked to carcinogenesis and reflect physiological changes in real time (15,16). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is widely used in metabolomics due to its high sensitivity – specificity, and ability to simultaneously quantify multiple metabolites, including bile acids, from small sample volumes (17).

This study aims to provide metabolomic insights into gastric cancer by profiling bile acids in plasma samples from GC patients and controls. By integrating advanced liquid chromatography-tandem mass spectrometry (LC-MS/MS) with comprehensive statistical analysis, this research wants to enhance the understanding of GC metabolism and contribute to the development of non-invasive diagnostic, prognostic tools and therapeutic targets.

## Materials and Methods

### *Patient characteristics and sample collection*

This study employed a case – control design to investigate bile acids metabolic alterations associated with gastric cancer (GC). The research was conducted in accordance with the principles of the Declaration of Helsinki and received approval from the Ethics Committee of Octavian Fodor Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania (approval no. 13656/22 December 2022), as well as from the Ethics Committee of Iuliu Haţieganu" University of Medicine and Pharmacy (approval no. 300/5 December 2022). Participant recruitment was carried out in a prospective manner between February 2023 and March 2024 in the Department of Surgery of IRGH in Cluj-Napoca, Romania.

Demographic characteristics and clinicopathological features of the study population are summarized in *Table 1*. The cohort included in the study comprised 62 adult patients diagnosed with early and invasive gastric cancer, stages I to III. A number of 35 patients received neoadjuvant FLOT chemotherapy, consisting of 5-fluorouracil, leucovorin, oxaliplatin, and docetaxel, administered in 4 to 8 cycles prior to biological sample collection. Neoadjuvant treatment was conducted in order to facilitate tumor downstaging, enhance surgical resectability, and prolong survival. For patients undergoing chemotherapy, biological samples were collected 4 to 6 weeks after the final treatment cycle. The control group included 70 individuals without evidence of gastric malignancy or other types of malignancies or chronic inflammatory diseases, matched to the gastric cancer group by age and sex to ensure demographic comparability.

**Table 1.** Demographic and clinicopathological data in gastric cancer group versus controls

| Demographics | GC Patients (n=62) | Control (n=70) |
|---|---|---|
| Sex (% males) | 67.7% | 68.6% |
| Median age (years) | 70 (57.8-74.0) | 70 (57.0-74.0) |
| Associated Conditions | | |
| Gastric ulcer | 30 (48.4%) | – |
| Atrophic gastritis | 42 (67.7%) | – |
| *H. pylori* infection | 32 (51.6%) | – |
| Lifestyle Habits | | |
| Smoking | 35 (51.6%) | – |
| Alcohol consumption | 15 (24.2%) | – |
| Gastric Cancer TNM Stage | | |
| Stage I | 13 (21.0%) | – |
| Stage II | 16 (25.8%) | – |
| Stage III | 33 (53.2%) | – |
| Tumor Histology (WHO classification) | | |
| Tubular adenocarcinoma | 34 (54.8%) | – |
| Signet ring cell carcinoma | 6 (9.7%) | – |
| Poorly cohesive carcinoma | 7 (11.3%) | – |
| Adenocarcinoma, mixed subtypes | 15 (24.2%) | – |
| Differentiation | | |
| Poorly differentiated | 27 (43.5%) | – |
| Moderately differentiated | 21 (33.9%) | – |
| Well differentiated | 14 (22.6%) | – |
| Treatment and Outcomes | | |
| Neoadjuvant chemotherapy | 35 (56.5%) | – |
| Surgery | 62 (100%) | – |
| Disease recurrence (6 months) | 21 (33.9%) | – |

Patients with acute inflammatory conditions, poorly controlled diabetes mellitus, severe heart failure (New York Heart Association class IV), advanced chronic kidney disease (stage 4 or higher according to the Kidney Disease Outcomes Quality Initiative classification), or a history of other malignancies within the preceding five years were excluded from the study.

Peripheral blood samples were obtained prior to surgical intervention and processed according to a standardized protocol. Blood was collected via venipuncture into sodium heparin – containing tubes and centrifuged at 1300 × g for 10 minutes at 4°C to separate plasma. The resulting plasma was aliquoted and stored at −80°C until further analysis.

### *Chemicals and Reagents*

Bile acids (BA) analytical standards of cholic acid (CA), glycocholic acid hydrate (GCA), sodium glycochenodeoxycholate (GCDCA), taurocholic acid sodium salt hydrate (TCA), sodium taurodeoxycholate hydrate (TDCA), sodium taurochenodeoxycholate (TCDCA), as well as the isotope-labeled BA used as internal standards (Bile Acids Standard Mixture pn. SMB00967) were purchased from Sigma-Aldrich (St Louis, MO, USA). The LC-MS grade methanol (MeOH) and acetonitrile (ACN) were purchased from VWR Chemicals (Leuven, Belgium). Ultrapure water was produced using the Elga Purelab water purification system (Elga Labwater, High Wycombe, UK). Formic acid (98-100%) was acquired from Merck (Darmstadt, Germany) and ammonium acetate from Sigma-Aldrich (Steinheim, Germany).

### *Sample Preparation*

A volume of 30 µL of plasma was processed from each sample. After adding the samples to MultiScreen®-HV 96-well plates with 0.45 µm hydrophilic Durapore® Membrane (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co Cork, Ireland), proteins were precipitated using 120 µL of methanol (containing BA internal standards), shaken for 1 minute at 600 rpm and room temperature, then filtered by centrifugation (2200 rpm for 5 min). The collected samples were subsequently dried under a stream of nitrogen ($N_2$) using a Microvap nitrogen evaporator (Organomation, Berlin, MA, USA). An additional purification step was done by redissolving the residue in methanol, centrifugation (2200 rpm for 5 min) and a second evaporation. The resulting samples were reconstituted in 30 µL of methanol before being subjected to LC-MS analysis.

### *LC-MS Analysis*

All analyses were performed using an LC-MS protocol described by Reiter et al. (17) with few modifications regarding the stationary phase and LC-MS instrument. The analyses were carried out using a Shimadzu Nexera Prominence LC (Shimadzu, Kyoto, Japan) coupled to a Sciex QTrap 4500 mass spectrometer (Sciex, Framingham, MA, USA), while the separation was achieved using a LUNA® Omega C18 stationary phase (100 mm × 2.1 mm ID, 1.6 µm, 100 Å) produced by Phenomenex (Torrance, CA, USA). All other used chromatographic and MS parameters were identical with those described in the above-mentioned reference. Instrument control, data acquisition and data processing were performed using the Sciex OS software (v 3.4.0). Two MS/MS transitions were recorded for each analyte. The quantity of each BA was calculated relative to the concentration of its internal standard.

### *Statistical Analysis and Machine Learning*

All statistical analyses were performed in R (R Foundation for Statistical Computing). Bile acid concentrations were analyzed after exclusion of

quality control samples. Metabolite variables with more than 15% missing values were removed prior to analysis to reduce the influence of sparsity. Remaining missing values were imputed using median imputation. Data were log10-transformed to stabilize variance and approximate normality, followed by autoscaling (mean centering and unit variance scaling) to ensure comparability across metabolites. Pairwise Pearson correlation coefficients were calculated among metabolites, and hierarchical clustering was performed using Ward's minimum variance method with a correlation-based distance metric ($1 - r$). Clustered correlation heatmaps and sample heatmaps were generated to visualize metabolite relationships and group-level patterns. To reduce multicollinearity, highly correlated variables were filtered using a correlation cutoff of 0.85. Among the remaining variables, group differences between control (CTR) and disease (GC) samples were assessed using two-sample Student's t-tests. Metabolites with $p < 0.05$ were considered statistically significant and retained for downstream analyses.

Principal component analysis (PCA) was performed on the selected metabolites using singular value decomposition of the scaled data matrix. Score plots (PC1 vs PC2) were used to evaluate sample clustering by group, while loading plots were examined to identify variables contributing to principal component separation. The cumulative variance explained by principal components was assessed using scree plots. Supervised classification was performed using linear discriminant analysis (LDA) implemented in the caret framework. Model performance was evaluated using repeated stratified 5-fold cross-validation (20 repetitions). Class probabilities obtained during cross-validation were used to generate receiver operating characteristic (ROC) curves, and classification performance was quantified by the area under the ROC curve (AUC).

### *Association of Bile Acid Profiles with Clinical and Pathological Variables*

Preprocessed bile acid data (log-transformed, median-imputed, and autoscaled as described above) were analyzed to investigate associations with demographic, clinical, and tumor-related variables in the gastric cancer (GC) cohort. Analyses were restricted to GC patients to evaluate disease-specific relationships. Associations between metabolite levels and continuous clinical variables (age and tumor dimension) were assessed using Spearman rank correlation, which does not assume normality and is robust to monotonic relationships. Correlation coefficients ($\rho$) and corresponding p-values were calculated for each metabolite independently. Metabolites with uncorrected $p < 0.05$ were considered statistically significant and were visualized using faceted scatter plots with linear regression overlays for visualization purposes only. Tumor stage variables (T stage, N stage, and TNM classification) were converted to ordinal numeric scales reflecting increasing disease severity, and correlations with metabolite levels were similarly evaluated using Spearman correlation. Correlation matrices were visualized using correlation plots, with non-significant associations ($p \geq 0.05$) masked.

### *Associations with Categorical Clinical Variables and Blood Biomarkers*

Associations between metabolite levels and binary categorical variables, including sex, smoking status, *Helicobacter pylori* infection status, and dysplasia, were evaluated using the Wilcoxon rank-sum test. Median differences between groups were calculated to estimate effect direction and magnitude. Metabolites with uncorrected $p < 0.05$ were visualized using faceted boxplots. Relationships between metabolite concentrations and laboratory parameters (including leukocytes, neutrophils, lymphocytes, hemoglobin, carcinoembryonic antigen, albumin, total protein, C-reactive protein, and modified Glasgow Prognostic Score) were assessed using Pearson correlation with pairwise complete observations. Correlation matrices were visualized using correlograms, with nonsignificant correlations ($p \geq 0.05$) omitted from the display.

## Results

### *Multivariate and Univariate Profiling of Plasma Bile Acid Pool*

High-resolution metabolic profiling demonstrated different plasma bile acid patterns between gastric cancer patients and healthy controls (*Fig. 1*).

Auto scaled concentrations presented variability in terms of distribution, while hierarchical clustering has grouped samples based on clinical status (*Fig. 1 A*). Pairwise Pearson correlation analysis demonstrated organized co-regulation among bile acid types, signifying coordinated connections within the bile acid pool (*Fig. 1 B*).

Univariate analysis identified a panel of metabolites significantly altered in gastric cancer group. The CA/CDCA ratio was reduced in cancer patients, together with significant differences in several secondary bile acids, including TLCA, GLCA, TDCA, GDCA, and GUDCA ($p < 0.05$; *Fig. 1 C*).

Principal component analysis (PCA) showed that

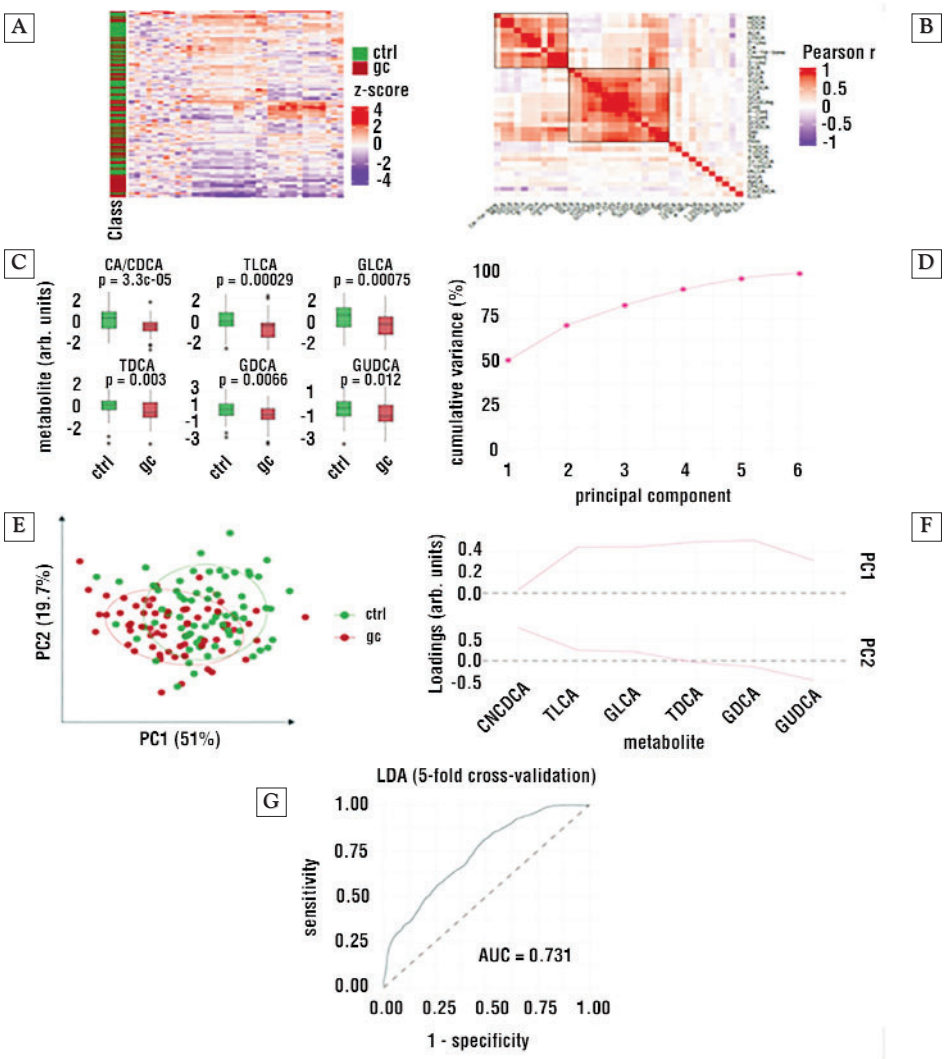


**Figure 1.** Multivariate and univariate analysis of bile acid profiles in gastric cancer versus control cohort
**(A)** Heatmap of autoscaled bile acid concentrations showing clustering of samples according to clinical group (control, ctrl; gastric cancer, gc); **(B)** Pearson correlation matrix of bile acids with hierarchical clustering, highlighting metabolite co-regulation patterns; **(C)** Boxplots of significantly altered metabolites between groups, including CA/CDCA ratio, TLCA, GLCA, TDCA, GDCA, and GUDCA (two-sample t-test p-values shown); **(D)** Scree plot displaying cumulative variance explained by principal components derived from selected metabolites; **(E)** Principal component analysis (PCA) score plot demonstrating partial separation between control and gastric cancer samples (PC1: 51%, PC2: 19.7% variance explained); **(F)** PCA loading profiles for PC1 and PC2 indicating metabolites contributing to group discrimination; **(G)** Receiver operating characteristic (ROC) curve from linear discriminant analysis (LDA) with 5-fold cross-validation, showing classification performance between groups (AUC = 0.731). Data were log-transformed, median-imputed, and autoscaled prior to analysis.

the first two components explained 70.7% of total variance (*Fig. 1 D–E*). The PCA score plot showed partial separation between GC and control samples, while loading metabolic profiles indicated that altered bile acids contributed substantially to group differences (*Fig. 1 F*).

Supervised classification using linear discriminant analysis (LDA) showed moderate discriminatory performance with an AUC of 0.731 (*Fig. 1 G*), indicating that plasma bile acid profiles differentiate gastric cancer patients from controls with measurable accuracy.

### *The Influence of Demographic and Clinical Factors on Bile Acid Profile*

Plasma bile acid profile was associated with demographic and clinical variables in gastric cancer group (*Fig. 2*).

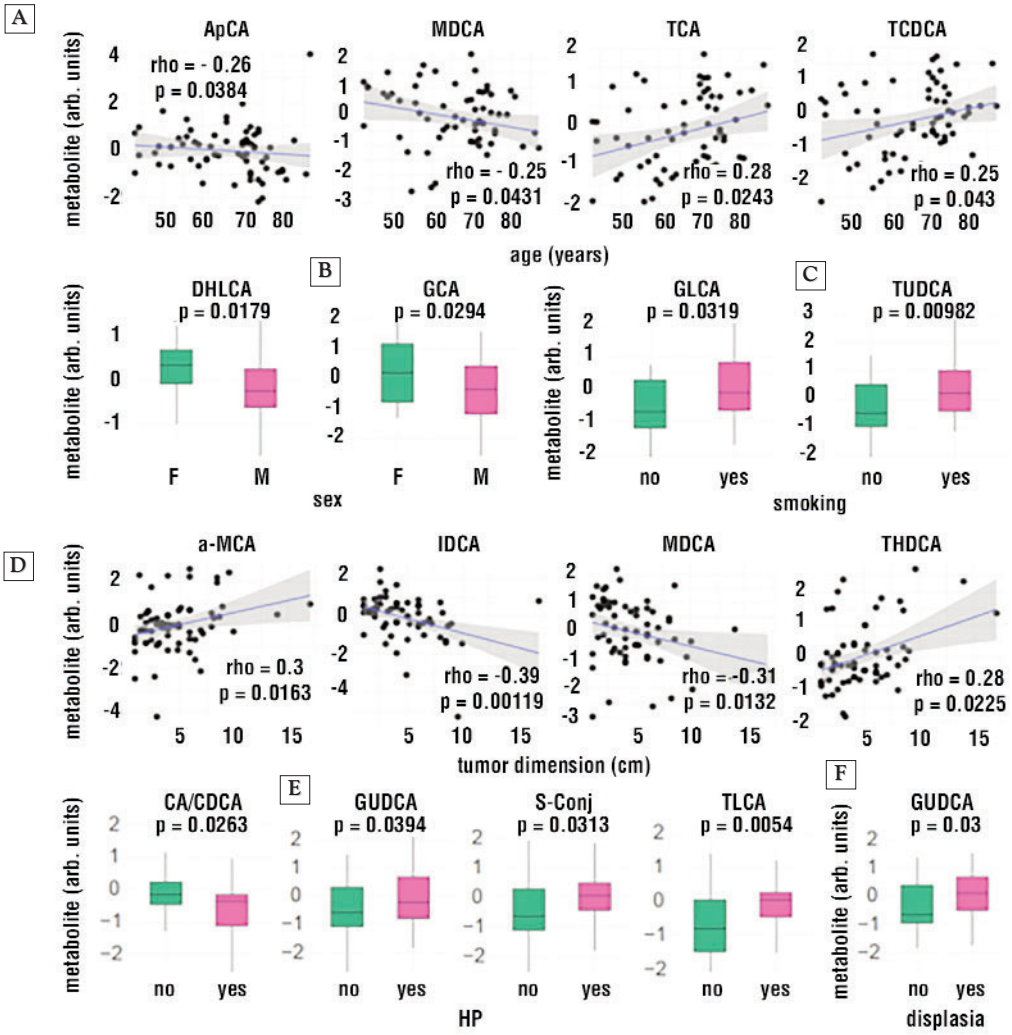


**Figure 2.** Associations between bile acid profiles and clinical variables in gastric cancer cohort
**(A)** Spearman correlation analysis between age and metabolite levels, showing significant associations for ApCA, MDCA, TCA, and TCDCA ($\rho$ and p-values indicated); **(B)** Sex-related differences in bile acid levels, with significant differences observed for DHLCA and GCA (Wilcoxon rank-sum test); **(C)** Smoking-associated alterations in GLCA and TUDCA levels (Wilcoxon rank-sum test); **(D)** Correlations between tumor dimension and metabolite concentrations, including a-MCA, IDCA, MDCA, and THDCA (Spearman correlation coefficients shown); **(E)** Differences in bile acid profiles according to Helicobacter pylori infection status, highlighting CA/CDCA ratio, GUDCA, secondary conjugated bile acids (S-Conj), and TLCA (Wilcoxon rank-sum test); **(F)** Association between dysplasia status and GUDCA levels (Wilcoxon rank-sum test).
Data represent log-transformed and autoscaled metabolite values. Statistical significance was defined as $p < 0.05$ (uncorrected).

Age exhibited negative association with ApCA ($\rho = -0.26$) and MDCA ($\rho = -0.25$), while taurine-conjugated bile acids (TCA and TCDCA) were positively associated with age (*Fig. 2 A*). Significant sex-related differences in plasma levels were observed for DHLCA and GCA ($p < 0.05$; *Fig. 2 B*). Smoking status was linked to modified GLCA and TUDCA levels (*Fig. 2 C*).

Tumor dimension correlated positively with α-MCA and THDCA, and negatively with IDCA and MDCA ($\rho = -0.39$ and $-0.31$, respectively; *Fig. 2 D*). *H. pylori* infection status was associated with differences in the CA/CDCA ratio, secondary conjugated bile acids (S-Conj), TLCA, and GUDCA (*Fig. 2 E*). In addition, GUDCA levels differed if dysplasia in the histopathological exam was present (*Fig. 2 F*).

### *Integration of Bile Acids with Systemic Laboratory Markers and Tumor Stage*

Spearman/Pearson correlation analysis (*Fig. 3*) revealed multiple significant associations between circulating

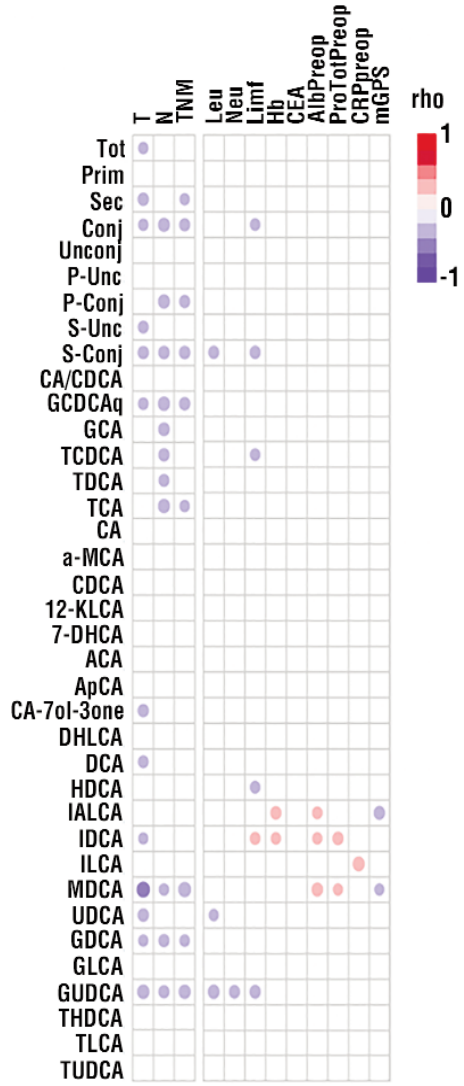


**Figure 3.** Correlation of bile acid profiles with clinical stage and blood biomarkers in the gastric cancer cohort

Correlation matrix showing associations between bile acid metabolites (rows) and clinical parameters (columns), including tumor stage variables (T, N, TNM) and laboratory markers (leukocytes, neutrophils, lymphocytes, hemoglobin, carcinoembryonic antigen, preoperative albumin, total protein, C-reactive protein, and modified Glasgow Prognostic Score). Spearman correlations were used for ordinal stage variables, and Pearson correlations for laboratory biomarkers. Correlation coefficients are represented by color intensity (red, positive; blue, negative). Only statistically significant correlations ($p < 0.05$, uncorrected) are displayed, with nonsignificant associations omitted. Data represent log-transformed and autoscaled metabolite values.

bile acids and clinical stage variables as well as laboratory biomarkers in the gastric cancer cohort. Tumor stage indicators (T, N, and TNM) exhibited primarily negative associations across different bile acid classes and individual metabolites, such as total bile acids, secondary bile acids, conjugated bile acids, and specific subsets of primary and secondary conjugated species, suggesting that more advanced disease correlates with reduced circulating bile acid levels. Moreover, other individual plasmatic metabolites, including GCDCA, TCDCA, TDCA, TCA, DCA, MDCA, UDCA, GDCA, and GUDCA, exhibited an inverse correlation with tumor stage characteristics, indicating a steady decline in bile acid levels concomitant with escalating tumor burden.

All indicators of systemic inflammation and immunological dysfunction exhibited inverse correlations with bile acid levels, indicating that increased inflammatory activity is related with reduced bile acid plasmatic levels. A subset of metabolites, specifically IDCA-, ILCA-, and MDCA-related species, exhibited positive relationships with hemoglobin, preoperative albumin, and total protein levels, suggesting elevated concentrations in patients with superior nutritional and physiological health. Significantly, several metabolites exhibited a negative association with the modified Glasgow Prognostic Score, representing that elevated bile acid levels confer a more advantageous systemic prognostic profile. Our data suggest that changes in bile acids in gastric cancer are significantly linked to tumor growth, systemic inflammation, and the nutritional status of the host, underscoring their potential as comprehensive metabolic markers of disease severity and patient condition.

## Discussions

Our targeted plasma metabolomic study demonstrates that resectable gastric cancer is associated with a distinct remodeling of the circulating bile acid pool, involving primary, secondary, and conjugated bile acid species. Among the 48 quantified plasma metabolites, the most relevant differences included a reduced CA/CDCA ratio and significant alterations in TLCA, GLCA, TDCA, GDCA, and GUDCA. Although bile acid profiling alone showed only moderate discriminatory ability between gastric cancer patients and controls, with an AUC of 0.731, these findings suggest that bile acid alterations may reflect a broader systemic metabolic phenotype rather than a single cancer-specific biomarker. The interpretation mentioned above is supported by Pan et al., who identified circulating bile acids as potential biomarkers for gastric cancer in a targeted metabolomics case–control study, and by Feng et al., who combined plasma metabolomics with fecal 16S rDNA sequencing in advanced gastric cancer and reported concomitant alterations in circulating metabolites and fecal microbiota (14,18). While Feng et al. focused on advanced gastric cancer, our findings indicate that bile acid-related metabolic alterations are also detectable in patients with resectable stage I–III disease.

A central finding of our study was the reduced CA/CDCA ratio in patients with gastric cancer. Since the relative abundance of cholic acid and chenodeoxycholic acid is influenced by hepatic bile acid synthesis and enterohepatic feedback regulation, this alteration may indicate a disturbance of host bile acid homeostasis in gastric cancer. However, this finding should not be interpreted as evidence of isolated enzymatic dysfunction, but rather as a circulating marker of altered gut–liver metabolic regulation. Zhong et al. demonstrated that reduced CYP8B1 activity in humans is associated with lower CA/CDCA ratios, supporting the biological relevance of this ratio as an indicator of bile acid synthetic pathway regulation (19). In addition, Fiorucci and Distrutti, as well as Monte et al., emphasized that bile acids are not only digestive molecules, but also bioactive mediators involved in metabolic regulation, inflammation, and gastrointestinal carcinogenesis (20,21). Therefore, the reduced CA/CDCA ratio observed in our cohort may represent part of a wider disruption of bile acid signaling and enterohepatic regulation in gastric cancer.

The significant changes observed in secondary and conjugated bile acids further suggest the involvement of microbiota-dependent bile acid metabolism. Secondary bile acids are generated through bacterial transformation of primary bile acids, while conjugated bile acid species are influenced by hepatic conjugation, intestinal deconjugation, microbial enzymatic activity, and enterohepatic circulation. Alterations in TLCA, GLCA, TDCA, GDCA, and GUDCA plasma levels may reflect changes in the interaction between host metabolism and microbial bile acid biotransformation. This interpretation is consistent with Jia et al., who described bile acid–microbiota crosstalk in gastrointestinal inflammation and carcinogenesis, and with Gou et al., who reviewed microbial metabolites in gastrointestinal cancers and highlighted bile acids among the metabolite classes linked to cancer-related biological processes (22,23). Similarly, Zeng et al. summarized evidence showing that alterations in the gastric microbiota and its interaction with *H. pylori* may contribute to gastric carcinogenesis (13).

The association between bile acid profiles and Helicobacter pylori status observed in our cohort further supports the possible link between chronic infection, mucosal injury, dysbiosis, and altered bile acid metabolism. In the present study, *H. pylori* infection status was associated with differences in the CA/CDCA ratio, secondary conjugated bile acids, TLCA, and GUDCA. These findings are in line with Duan et al., who described *H. pylori*-associated gastric carcinogenesis as a multifactorial process involving chronic inflammation, epithelial injury, immune modulation, and alterations in the tumor microenvironment (24). Moreover, Zhang et al. recently reviewed the bidirectional crosstalk between bile acids and gut microbiota in precancerous gastric lesions, emphasizing the potential relevance of bile acid dysregulation during the transition from chronic mucosal injury to gastric carcinogenesis (10). Therefore, the associations identified in our study suggest that infection-related changes in the gastric environment may have systemic metabolic correlates reflected in circulating bile acid profiles. Nevertheless, because of the fact that gastric microbiota sequencing and bile reflux assessment were not performed in this study, these results should be interpreted as indirect evidence requiring further validation.

Beyond group discrimination, our data indicate that circulating bile acids are associated with tumor burden and clinico-pathological characteristics severity. More advanced T, N, and TNM stages showed predominantly inverse associations with total bile acids, secondary bile acids, conjugated bile acids, and several individual metabolites, including GCDCA, TCDCA, TDCA, TCA, DCA, MDCA, UDCA, GDCA, and GUDCA. These findings suggest that gastric cancer progression may be accompanied by a progressive disruption of bile acid metabolism. Pan et al. also reported significant alterations in the circulating bile acid pool between gastric cancer patients and controls, as well as correlations between bile acids and clinical traits (18) and Feng et al. reported that metabolic alterations and fecal microbiota changes coexist in advanced gastric cancer, supporting the concept that disease progression is associated with broader host metabolic and microbial disturbances (14). Our study extends this perspective to a surgically treated stage I–III cohort and suggests that circulating bile acid abnormalities may already be present before resection and may correlate with disease severity.

The correlations between bile acid concentrations and systemic inflammatory, nutritional, and hematological markers further support the interpretation of bile acids as integrated indicators of host condition. In our cohort, bile acid levels were negatively associated with inflammatory and prognostic markers such as CRP and the modified Glasgow Prognostic Score, while selected metabolites were positively associated with albumin, total protein, and hemoglobin. This pattern suggests that reduced circulating bile acid concentrations may accompany the inflammatory and catabolic state frequently observed in patients with gastric cancer. McMillan described the Glasgow Prognostic Score as a systemic inflammation-based prognostic marker in cancer, while Gao and Huang reviewed its value specifically in gastric cancer (25,26). More

recently, Melekoglu et al. reported that an elevated pretreatment modified Glasgow Prognostic Score was associated with poorer survival in elderly gastric cancer patients treated with perioperative FLOT, supporting the clinical relevance of inflammation-based nutritional indices in this setting (27).

### *Study Limitations*

This study has several limitations that should be acknowledged. It is a case–control design and this limits the ability to establish causal relationships between bile acid alterations and gastric cancer, and the observed findings should therefore be interpreted as associative. Second, this was a single-center study including 62 patients with resectable stage I–III gastric cancer and 70 matched controls, which may limit the general aspect of the results to larger and more diverse populations, as well as to patients with metastatic or unresectable disease. Also, substantial proportion of patients received neoadjuvant FLOT chemotherapy before sample collection, and although blood samples were obtained 4–6 weeks after the final treatment cycle, residual effects on systemic metabolism, inflammation, nutritional status, and circulating bile acid concentrations cannot be excluded. Nevertheless, the study focused exclusively on plasma bile acid profiles and did not include paired gastric tissue, bile, fecal samples, or microbiome sequencing; therefore, the proposed involvement of the gut–liver–microbiome axis remains indirect and requires further validation. Bile acid profiling alone demonstrated moderate discriminatory performance, supporting its potential role as part of integrated biomarker panels rather than as an independent diagnostic tool.

## Conclusions

A reduced CA/CDCA ratio and significant, consistent alterations in secondary and conjugated bile acids (TLCA, GLCA, TDCA, GDCA, and GUDCA) are significantly implicated in gastric carcinogenesis. Bile acid dysregulation in gastric cancer appears to reflect systemic metabolic alterations rather than a strictly localized disturbance, potentially involving the gut–liver–microbiome axis. Helicobacter pylori chronic infection suggests a link between ongoing mucosal damage, an imbalance in the microbial environment, and changes in how bile acids are processed.

Bile acid profiling can become clinically relevant as comprehensive metabolic indicators, by exhibiting potential as components of multimodal biomarker panels, particularly for assessing host metabolic status.

### *Author's Contributions*

Conceptualization, Ş.U., R.A.C., N.A.H., C.D.G. and F.Z.; methodology, Ş.U., A.C., T.M., R.C.M. and A.I.B.; investigation, Ş.U., C.P.U., F.Z., C.I.B., R.A.C. and Z.S.; formal analysis and data curation, R.C.M., T.M., A.I.B., R.S.P. and C.A.I.; writing – original draft preparation, Ş.U., R.A.C.; writing – review and editing, all authors; supervision, C.A.I., C.D.G. and N.A.H.; project administration, Ş.U., R.A.C. and N.A.H.; funding acquisition, Ş.U. and N.A.H.

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### *Conflicts of Interest*

The authors declare no conflict of interest.

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### *Institutional Review Board Statement*

This study was conducted in accordance with the Declaration of Helsinki of 1964 and later amendments and was approved by the Ethics Committees of Octavian Fodor Regional Institute of Gastroenterology and Hepatology in Cluj-Napoca (no. 13656, approval date: 22.12.2022) and "Iuliu Haţieganu" University of Medicine and Pharmacy (no.300, approval date: 5.12.2022).

### *Informed Consent Statement*

Informed consent was obtained from all participants included in the study.

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