

Serum metal profiles and gallbladder cancer risk in an Indian population: Evidence from a Case-Control Study

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Abstract

Gallbladder cancer (GBC), a rare yet aggressive biliary tract malignancy, varies by geographic location, with greater incidence in the north and northeast of India along the Gangetic belt, suggesting that environmental risk factors may contribute to disease etiology. A total of 912 GBC cases and 1140 visitor controls were enrolled at Tata Memorial Centre, India. The concentration of non-essential (Cr, Ni, As, Cd, Pb, U) and essential metals (Mn, Cu, Se) were measured using the inductively coupled plasma mass spectrometer (ICPMS). Individual metal effects were presented using logistic regression model adjusting for confounders, while joint metal mixture effects were obtained using weighted quantile sum and quantile g-computation methods. A strong J-shaped non-linear association for Cu (OR = 10.21; p-value $\leq$ 0.005) and Cd (OR = 4.79; p-value $\leq$ 0.005) was observed. The strongest inter-metal correlation was observed for Mn-U. Exposure to metal mixture showed one-unit increase in the WQS index with increased GBC risk (OR = 6.19; p-value < 0.00001), with the highest weight contributed by Cu (~ 60%), followed by Cd (~ 27%) and Mn (~ 13%). Metal mixtures play a significant carcinogenic role in the development of GBC. Identification of the source of exposure, particularly in the high-risk areas of the GBC, would be useful in primary prevention.

Introduction

Gallbladder cancer (GBC) is a biliary tract malignancy that accounts for 80–95% of the biliary tract cancers globally.¹ It ranks sixth amongst the gastrointestinal cancers¹ with 1.2 global age-standardized incidence rate (ASIR) and age-standardized mortality rate (ASMR) of 0.83 per 100,000 for GBC². The highest incidence rates are reported in South America, South Central Asia and Eastern Asia.² India accounts for 10% of the global GBC burden³, with an ASIR of 1.5 and ASMR of 1.8 per 100,000 reported in 2022, and a five-year prevalence of 2.6%.⁴

India exhibits significant geographic variation in GBC incidence with the highest rates reported in the central, northern and north-eastern regions,⁵ particularly within the Gangetic belt – in the states of Bihar, Uttar Pradesh, Jharkhand, Uttarakhand and West Bengal.⁶ This regional clustering suggests a role for environmental and population-specific risk factors. In addition to the known risk factors,^{7–11} environmental exposure to metals poses a significant yet lesser-explored etiological risk factor for the development of GBC.

Exposure to metals may disrupt biological processes such as oxidative stress, inflammation, and altered cellular homeostasis. Essential trace elements such as copper (Cu), manganese (Mn), and selenium (Se) play important roles in physiological processes, but imbalances may disrupt redox regulation and cellular function.^{12,13} Non-essential/toxic metals such as cadmium (Cd) and lead (Pb) have no known biological role and are toxic even at low concentrations.

Several studies in India have reported the association between metal exposure and GBC, including arsenic (As) contaminated groundwater and elevated concentrations of Cd, chromium (Cr), nickel (Ni)

and Pb in drinking water in high-risk regions.^{14–16} Additionally, studies from China and Chile have measured metals in serum and tissue specimens, reporting altered metal concentrations in GBC patients.^{17,18}

Although Indian studies have examined the relationship between metals and GBC^{19–23}, these studies are limited by small sample sizes (often < 160 GBC cases), focus on individual metals rather than combined exposures, limited adjustment for established confounding risk factors⁷ and less sensitive exposure measurement methods.

To address these gaps, we conducted a large hospital-based case–control study in an Indian population to evaluate the association between serum metal profiles and GBC risk, with particular focus on both essential and non-essential elements individually and along with their combined mixture effects. Serum concentrations of non-essential elements - As, Cr, Cd, Pb, Ni and uranium (U) and essential trace elements – copper (Cu), manganese (Mn), and selenium (Se) were quantified by a highly sensitive inductively coupled plasma mass spectrometer (ICPMS).

Methods

Study Design:

We conducted a hospital-based case-control study at the Tata Memorial Hospital (TMH), Mumbai, from September 2010 through June 2015. The study enrolled 912 GBC cases and 1140 visitor controls. The inclusion criteria for GBC cases were as follows: histopathologically and cytologically confirmed primary GBC or using clinical diagnostic imaging such as abdominal ultrasound and/or abdominal computed tomography and/or magnetic resonance imaging from the gallbladder and histological/cytological findings from the metastatic site with a date of diagnosis less than one year from the date of enrolment. Cases were excluded if they had neoplasms arising from the ampulla of Vater (C24.1), overlapping lesions of the biliary tract (C24.8), unspecified biliary tract lesions (C24.9), or any other malignancies. Visitor controls with no history of cancer that accompanied the case participants to TMH and ACTREC were uniformly enrolled from all the disease management groups (DMGs: structured programs to improve patient care with chronic conditions), and did not exceed 30% for an individual control DMG (**supplementary table S1**). At the time of enrolment, controls were frequency-matched to cases based on age (± 10 years), sex and current residential region, categorizing them into five regions of India. Eligible participants were Indian residents for at least one year and stratified into five age groups (< 30, 30-39, 40-49, 50-59 and >60 years) (**supplementary table S2**). Pregnant or lactating women were excluded from both cases and controls.

Epidemiological data acquisition and assessment:

Trained interviewers collected data using a detailed questionnaire gathering information about the age, sex, body mass index (kg/m^2), birth region, current residential region, history of any tobacco use

(chewing/smoking), self-reported history of gallstones, mustard oil consumption and education (**supplementary table S2**). The reproducibility of the questionnaire was assessed using an abbreviated version administered at least seven days after the initial interview. For this, we re-interviewed 3% of the total enrolled participants with a 100% agreement percentage for sex, 97.67% for smoking and 95.35% for tobacco chewing.

Biospecimen collection and fractionation:

With individual consent from the study participants, a total of 5 mL of whole blood was collected by trained phlebotomists using standard operating protocols for blood collection in a single serum separator tube (BD vacutainer®). The sample tubes were centrifuged at 4000 rpm for 20 mins at 4° C before storing the respective fractions in a Biobank at -80°C or liquid nitrogen until further analysis.

Serum Metal analysis:

Serum samples were subjected to controlled, stepwise thawing followed by elemental analysis using Thermo Scientific™ iCAP™ TQ (triple quadrupole) ICPMS equipped with a collision reaction cell (CRC). Metal quantification was carried out from December 2024 to October 2025, using archived biospecimen maintained under appropriate storage conditions. Concentrations of nine metals (Cr, Ni, As, Cd, Pb, U, Mn, Cu and Se) were simultaneously measured and reported in micrograms per litre (µg/L) using the Qtegra™ software (Version: 2.14.5122.467)²⁴. A seven-point calibration curve was used for target analytes ranging from 0.01µg/L to 100µg/L with an acceptable correlation coefficient (R^2) of ≥ 0.995 (**supplementary table S3**). A batch sequence included 12 cases and 15 control samples, along with 7 calibration standards, 8 flanking method blanks between sample sets, two sets of certified reference materials (CRMs) - ClinCal® serum calibrator, and ClinChek® Level I and Level II, (Catalog numbers: 9982, 8880, 8881, M/s Recipe, GmbH, Munich, Germany). Cases and controls were analysed within each set to reduce analytical bias. Stringent quality control checks like intra-assays (n=228), inter-assays (n=107) and a drift standard were evenly distributed throughout each batch to ensure accuracy and reproducibility of the results. Method detection limit (MDL) for each metal was calculated using the mean value of all method blanks (n=608). The MDL value for each metal was calculated using the weighted average method using individual MDL values across all analytical days (n=76). All values below MDL were replaced with the MDL/√2 value.^{25,26} To address outliers of each metal, the percentile distribution for cases and controls was calculated, and the 99th percentile value of the control group was used to replace the outliers across both groups. As the data distribution was asymmetric, we log-transformed the outlier-replaced data.

A detailed description of the laboratory protocols, instrument parameters, quality control checks (intra- and inter-assay coefficients of variation, intraclass correlation coefficient, CRMs, drift standard), MDL and percentage of samples falling below the detection limit is provided in the supplementary tables (**supplementary tables S4-S19**).

Statistical Analysis:

Participant characteristics were summarized by case–control status and sex using mean and standard deviation (SD) for continuous variables and frequencies (%) for categorical variables. Group differences were assessed using Student's t-test²⁷ and chi-square test²⁷.

Spearman correlation analysis²⁸ was used to assess inter-metal relationships using original metal concentration data. Sensitivity analyses were conducted for metals showing moderate to strong correlation with a similar method used in the multivariate logistic regression described subsequently.

Hierarchical clustering based on a correlation-derived distance matrix ($1 - \text{correlation}$) and Ward's method²⁹ was applied to identify similarities in metal profiles between cases and controls. Similarities between clusters are interpreted based on the height of the vertical line between each metal cluster. The branches joining at a lower height indicated a strong similarity, whereas those showing a greater height were indicative of weak similarity.

Associations between serum metal concentrations and GBC risk were evaluated using multivariable unconditional logistic regression.³⁰ Log-transformed metal concentrations were categorized into tertiles based on the control distribution, with values below the MDL imputed as $\text{MDL}/\sqrt{2}$ and upper outliers replaced by the 99th percentile value of the control group. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated, adjusting for age (continuous), sex (male and female), residential risk region (high and low risk), gallstones (Yes/No), any tobacco use (Ever-Never used), mustard oil consumption (Ever-Never used), and education (≤ 5 years and > 5 years of schooling). Bonferroni correction was applied to account for multiple comparisons ($p \leq 0.005$).³¹

Stratified analysis was performed across confounders like sex, birth risk region (high and low-risk), and any tobacco use (Ever-Never used). Interaction of confounding variables with metal concentration was studied using the likelihood ratio test (LR test) to estimate a P-value for interaction,³² and using continuous log-transformed metal values, the P-value for trend was estimated for the risk due to the metals, for all the strata.

Non-linear dose–response relationships between serum metal concentrations and GBC risk were evaluated using restricted cubic spline models (rms package) with four knots placed at the 5th, 35th, 65th, and 95th percentiles³³. Analyses were conducted on MDL-imputed ($\text{MDL}/\sqrt{2}$) and outlier-replaced (99th percentile value of the control group) log-transformed data, with multivariable adjustment for confounders. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated. Statistical significance of overall associations and non-linearity was assessed using RCS ANOVA (P-overall and P-nonlinear), with Bonferroni-corrected significance ($p \leq 0.0005$).

Metal mixture effects on GBC risk were evaluated using weighted quantile sum regression (gWQS package, R).³⁴ Metal concentrations were categorized into quantiles, and data were split into training (60%) and validation (40%) sets. The training set was used to estimate metal weights via bootstrap sampling (1,000 iterations), reflecting each metal's contribution to the mixture. A weighted index (sum of

weight $\times$ quantile)³⁵ was constructed and applied to the validation set to estimate associations with GBC using logistic regression, adjusting for the same covariates as in single-metal models.³⁶

The quantile g-computation (qgcomp package, R)³⁷ method was utilized to study the joint effect of metal co-exposure on GBC risk. Metal concentrations were categorized into quartiles, and models were adjusted for the same covariates as in multivariable logistic regression. The overall mixture effect was estimated using the ψ parameter, representing the change in log-odds of GBC per one-quartile increase in all exposures. Nonparametric bootstrapping (1,000 iterations) was used to derive standard errors and 95% confidence intervals.³⁸ Directional weights were calculated to quantify the relative contribution of each metal to the mixture effect.³⁸ All analyses were performed using Stata version 15.0³⁹ and R version 4.5.1. Additional methodological details are provided in the supplementary Information.

Ethics Approval:

Informed consent was obtained before enrolling the study participants. This research was approved by the Institutional Ethics Committee of Tata Memorial Hospital, Mumbai, with the corresponding Institutional Review Board project approval numbers: 368 and 3065, and was conducted in strict compliance with the Declaration of Helsinki.

Consent to Participate:

Participants were informed about the nature, purpose and any potential risks of the study to ensure clarity of the research process. Informed consent forms provided in the local language allowed the participants to make well-informed decisions for their study involvement. Each participant was given the opportunity to ask questions and address any concerns prior to providing written consent.

Results

The case-control participant characteristics stratified by sex are summarized in **Table 1**. The mean age of GBC cases was ~50 years ($\pm$ SD 10.11), compared with ~47 years ($\pm$ SD 10.21) for controls. Females accounted for 68% of cases and 82% of controls. Around 40% of the GBC cases self-reported a history of gallstone as compared to 3% of the controls. A history of gallstone was reported in 44% of the female cases as compared to 31% for the male counterpart. GBC cases showed a greater use of tobacco intake (31%) and mustard oil consumption (78%) compared to controls (21% and 50%, respectively) (**Table 1**). For GBC cases, 17% of females showed use of tobacco as compared to 61% of males. An increased mustard oil consumption was recorded in the female GBC cases (80%) as compared to the male cases (74%). With respect to the educational status, 77% of the control population showed ≥ 5 years of education compared to 58% in the GBC case group.

The summary statistics for metal showed elevated mean concentrations for Cd (~4 μ g/L), Mn (~7 μ g/L) and Cu (3200 μ g/L) in cases compared to the control group. An increase in metal concentrations was observed from the 25th percentile distribution in GBC cases compared to controls, except for Se and U.

Cr showed a majority of samples below MDL (68%) followed by Pb (57%) and Cd (40%) (**supplementary table S19**).

Spearman correlation matrix showed a very strong correlation between U - Mn ($r = 0.80$) and moderate correlations between Cd - Mn ($r = 0.52$) and Cr - Ni ($r = 0.48$) within cases. In controls, moderate correlations were observed between Cr - Ni ($r = 0.51$), U - Mn ($r = 0.52$), Cd - Mn ($r = 0.57$), Pb - Mn ($r = 0.48$) and Ni - Mn ($r = 0.45$). (**supplementary figure S1**).

Upon exploring the cluster analysis individually in cases, there were two clusters observed – the first cluster involving Cr, Ni, Pb, Se and the second cluster had Mn, U, Cd, As, & Cu. Within this, Cr-Ni and Mn-U showed greater similarity in clustering patterns. Controls showed four specific clusters with Cu-Se, Cr-Ni and Mn-Cd showing a significant similarity at a low linkage distance, whereas As reveals an independent second cluster before joining other metals (**supplementary figure S2**).

Multivariate unconditional logistic regression showed a significant increase in the risk of GBC except Ni, As and Pb. Among non-essential metals, Cd showed a dose-response 5-fold increased risk for GBC in the highest tertile (OR_{Cd} : 4.79, 95%CI: 3.70-6.21, $P_{\text{bonferroni}} \leq 0.005$). The metal concentrations falling in the highest tertile of Cr (OR_{Cr} : 1.55, 95%CI: 1.23-1.96, $P_{\text{bonferroni}} \leq 0.005$) and U (OR_U : 1.69, 95%CI: 1.29-2.22, $P_{\text{bonferroni}} \leq 0.005$) showed an increased risk for GBC. (**Table 2**). Among essential metals, Cu showed a 10-fold increased risk for GBC in the highest tertile (OR_U : 10.21, 95%CI: 7.31-14.27, $P_{\text{bonferroni}} \leq 0.005$), whereas Mn concentrations showed a dose-response risk (OR_{Mn} : 4.88, 95%CI: 3.58-6.67, $P_{\text{bonferroni}} \leq 0.005$).

In the sex-stratified analysis, females showed a significant association of GBC risk for the highest tertiles of Cr (OR_{Cr} : 1.57, 95%CI: 1.19-2.09, $P_{\text{bonferroni}} \leq 0.005$), Cd (OR_{Cd} : 8.95, 95%CI: 6.41 - 12.50, $P_{\text{bonferroni}} \leq 0.005$), Mn (OR_{Mn} : 7.05, 95%CI: 4.86- 10.24, $P_{\text{bonferroni}} \leq 0.005$) and Cu (OR_{Cu} : 4.64, 95%CI: 3.15-6.84, $P_{\text{bonferroni}} \leq 0.005$) with significant p-trend values ($p\text{-trend} \leq 0.005$). However, Cu depicted a different direction of association for males, showing an extremely high GBC risk (OR_{Cu} : 58.49, CI: 27.58-124.05, $P_{\text{bonferroni}} \leq 0.005$) compared to females (**supplementary table S20**).

Birth risk region (high-risk and low-risk) based stratified analysis resulted in a similar trend across all metals except Se & As, with a significant difference of association for the highest tertile of Cd (OR_{Cd} : 7.10, 95%CI: 3.98-12.66, $P_{\text{bonferroni}} \leq 0.005$), Mn (OR_{Mn} : 11.52, 95%CI: 5.10-26.00, $P_{\text{bonferroni}} \leq 0.005$) and Cu (OR_{Cu} : 13.32, 95%CI: 6.04-29.40, $P_{\text{bonferroni}} \leq 0.005$) in the low-risk region compared to the high-risk region (**supplementary table S20**).

In stratified analysis based on any tobacco use, Cd (OR_{Cd} : 6.21, 95%CI: 4.55-8.49, $P_{\text{bonferroni}} \leq 0.005$), U (OR_U : 2.27, 95%CI: 1.65-3.12, $P_{\text{bonferroni}} \leq 0.005$), and Mn (OR_{Mn} : 5.63, 95%CI: 3.93-8.07, $P_{\text{bonferroni}} \leq 0.005$) showed a positive association in the highest tertile of the non-user group as compared to the user group. Cu showed an 18-fold increased risk for GBC in the user group (OR_{Cu} : 18.44, 95%CI: 9.71-

35.02, $P_{\text{bonferroni}} \leq 0.005$) compared to an 8-fold risk in the non-user group (OR_{Cu} : 8.35, 95%CI: 5.58-12.50, $P_{\text{bonferroni}} \leq 0.005$), suggesting a risk difference of ten-fold (**supplementary table S20**).

However, for Se, all the stratified ORs at the highest concentrations reported a significant inverse association and p-trend value, thus suggesting its possible role in decreasing the risk of GBC among all stratified groups (**supplementary table S20**).

A J-shaped curve was observed for Cu and U ($p\text{-overall} < 0.0001$; $p\text{-nonlinear} < 0.0001$), presenting a significant non-linear association with GBC risk. Cd ($p\text{-overall} < 0.0001$; $p\text{-nonlinear} < 0.0121$) and Mn ($p\text{-overall} < 0.0001$; $p\text{-nonlinear} < 0.0499$) showed a non-linear trend yet statistically insignificant risk for GBC. Cr and Ni showed a significant non-linear yet decreased risk for GBC. Pb and As showed an insignificant association with GBC. Se showed an inverted J-shaped curve with a significant non-linear response, presenting a decreased GBC risk at higher serum concentrations. (**Figure 1**).

WQS regression demonstrated a significant positive association between heavy metal mixture exposure and GBC risk (**Figure 2A**), with a one-unit increase in the WQS index being associated with increased odds of GBC (OR : 6.19, 95%CI: 4.84-7.95, $p < 0.00001$). A clear monotonic dose-response relationship was observed, with increasing WQS index corresponding to a higher predicted probability of GBC, indicating a consistent adverse effect of the metal mixture. The cluster points above the curve line graph represent the distribution of cases, while the ones below represent the distribution of control.

The relative contribution of individual metals to the combined metal mixture risk is presented in **Figure 2B**. The threshold value for the relative weights was predefined based on the number of metals being included in the mixture analysis ($1/9 \approx 11\%$), and metals exceeding this threshold were significant contributors to the metal mixture risk. Thus, Cu exhibited the highest weight ($\sim 60\%$), followed by Cd ($\sim 27\%$) and Mn ($\sim 13\%$), all exceeding the predefined threshold, while other metals contributed minimally.

Qgcomp analysis demonstrated a significant positive association between the metal mixture co-exposure and GBC risk (OR : 1.35, 95% CI: 1.23- 1.47; $p < 0.00001$), indicating that a one-quartile simultaneous increase in all metal exposures was associated with an increase in the log-odds of GBC (**Figure 2C**).

Upon gender stratification, WQS analysis showed an increased odds of association for GBC risk in females (OR : 8.01, 95% CI: 5.81-11.03; $p < 0.001$) as compared to males (OR : 7.12, 95% CI: 4.48-11.32; $p < 0.001$). Qgcomp analysis showed a significant positive association for the risk for developing GBC in females (OR : 1.43, 95% CI: 1.29-1.59; $p < 0.001$) compared to males (OR : 1.16, 95% CI: 1.03-1.30; $p < 0.016$) (**supplementary table S22**).

Directional weight analysis revealed that Cu, Mn, Cd, and Cr contributed positively within the metal mixture, whereas Se, U, Ni, Pb, & As exhibited negative weights, suggesting an inverse association with GBC risk (**Figure 2D**).

The sensitivity analysis for highly correlated metals (Cd, Mn and U) showed a significant association for Cd (OR_{Cd} : 3.23, 95%CI: 2.40-4.34, $P_{\text{bonferroni}} \leq 0.005$) in the highest tertile with a dose-response gradient. Mn showed a 3-fold increased risk for GBC in the third tertile (OR_{Mn} : 3.09, 95%CI: 2.13-4.48, $P_{\text{bonferroni}} \leq 0.005$) whereas U showed an insignificant association. (**supplementary table S21**).

Summary statistics for the metal concentrations, vital metrics including the minimum, maximum, percentile values, mean and SD of the concentrations, Limit of Quantification (LOQ)²⁵, and MDL can be found in **supplementary table S20**.

Discussion

In our large case-control study, we show that serum metal profiles are strongly associated with GBC, with positive associations for Cd, Cu, Mn, and U, and an inverse association for Se. Importantly, these associations were non-linear for Cd, Cu, Se and U, with Cu and Cd contributing the largest share to the overall mixture effect. Unlike prior studies that evaluated individual metals, our findings demonstrate that GBC is shaped by combined metal exposures with a non-linear exposure-response association.

Our findings align with and extend prior evidence linking metal exposure to GBC. A large population-based metallomic analysis by Lee et al conducted in China demonstrated that serum metals are associated with both gallstones and GBC, with positive associations for Cd and Cu, inverse associations for Se, and consistent non-linear exposure-response relationships across the disease continuum (**supplementary table S23**).¹⁷ Earlier studies from India have reported concordant evidence, particularly for the high-risk regions within the Indo-Gangetic belt. Shukla et al. reported markedly elevated Cd, Cr, and Pb concentrations in the bile of GBC patients, indicating local hepatobiliary accumulation of toxic metals¹⁹. Basu et al. further showed that these alterations extend to systemic and tissue compartments, with reduced Se, zinc (Zn) and increased Cu, Cd, Cr, Pb, and Ni across serum, bile, and gallbladder tissue²⁰. A recent study by Nelson et. al reported increased concentrations of Mn, Cu and Zn within blood and urine samples, highlighting their positive correlation with the biliary pathology [GBC and Gallstone disease (GSD)].²²

Our findings, based on a large Indian case-control study, provide corroborative evidence for Cd, Cu, Mn and Se, but extend prior work by demonstrating that GBC could be driven by metal mixtures rather than individual elements. We further quantify the relative contribution of each metal and identify structured metal-co-exposure patterns, including clustering of Mn, Cd, and U, suggesting shared environmental or geochemical sources relevant to high-risk regions.

The observed co-exposure structure provides additional insight into the mixture-driven nature of GBC risk. Correlation and clustering analyses showed strong and consistent co-existence among Mn, Cd, and U, as well as distinct clustering patterns between cases and controls. Interestingly, this converges with the geochemical behaviour of U⁴⁰, which shows higher adsorption to Mn and iron (Fe)⁴¹ present in the soils. These findings suggest that metals may originate from shared environmental sources

(**supplementary table S24**) or follow linked biological pathways, supporting the use of mixture-based approaches over single-metal models.

Cu, Cd, and Mn showed cross-method concordance across WQS and Qgcomp models, further strengthening their role as key drivers of GBC risk. Differences in directional weights across both models highlight the complexity of interpreting individual metals within a correlated exposure matrix.

Non-linear exposure–response association was one of the central features of our findings and provides important biological context. The J-shaped associations observed for Cu and U suggest that both low and high exposure levels may have distinct effects, while the inverted J-shaped association for Se is consistent with its role as an essential element with protective effects at higher concentrations. These patterns are consistent with homeostatic regulation of essential metals and threshold effects for toxic metals and indicate that linear models may not adequately capture the relationship between metal exposure and GBC risk.

Differences between our findings and earlier studies likely reflect biospecimen-specific limitations. Cr and Pb showed weak or non-significant associations in serum, despite strong positive associations in whole blood, bile and tissue in previous work^{19,20,22}(**supplementary table S25**). This is expected given that Pb is predominantly bound to erythrocytes and Cr is present at low concentrations in serum due to rapid clearance and limited protein binding.⁴² Similar considerations apply to As and Ni, which have short serum half-lives (Ni: up to 48 hours, As: three to five days)^{43,44} and require appropriate biospecimens or speciation approaches for accurate As exposure. A study by Palma et al. measured As species – AsIII, AsV, MMA, DMA and AsB in patients with GBC compared to NCGB (non-cancerous gallbladder).

Our case-control study presents a major constraint in the collection of relevant biospecimens (spot urine, bile) mainly due to individual variability in urine concentrations due to hydration levels and invasive methods for bile collection, which is difficult to attain in recruiting healthy controls. Hence, only serum samples were analysed in the present study, whereas GBC tissue samples will be utilised for exposure assessment in future studies.

However, reported serum concentrations of Cd and Pb in a prior study²⁰ are substantially higher than those observed in population-based biomonitoring. In that study, mean serum Cd (~6.5 µg/L) and Pb (~40 µg/L) exceeded the levels in our cohort (Cd: 0.04–0.09 µg/L; Pb: 0.73–0.86 µg/L) by approximately one to two orders of magnitude. These values are also well above typical serum concentrations reported in general populations, wherein the permissible limit for Cd is usually <0.2 µg/L and for lead <1 µg/L.⁴⁵ Given the established distribution of Pb in blood, a serum concentration of ~40 µg/L would imply exceptionally high systemic exposure. Such discrepancies may reflect differences in analytical methods, sample handling (e.g., hemolysis), unit reporting, or potential contamination, and highlight the importance of rigorous quality control in trace metal measurements.

Tobacco use, a known source of Cd and Pb exposure, and differences in socioeconomic status may further contribute to variation in metal levels.^{46–48} However, the persistence of strong exposure–response relationships after adjustment for these variables, particularly for Cd, Mn (~5-fold risk), and Cu (~10-fold risk), suggests that confounding alone is unlikely to explain the observed associations. These findings suggest that while environmental and lifestyle factors determine exposure levels, the observed associations reflect biological effects of metal accumulation and changes in metal homeostasis. This interpretation is further supported by the differential direction of associations across multiple metals.

The strong association observed for serum Cu supports a role for disrupted metal homeostasis in carcinogenesis. Cu is tightly regulated under physiological conditions but is frequently elevated in malignancy (cuproplasia), where it promotes cell proliferation and metabolic activity^{49,50}. In our study, Cu accounted for the largest proportion of the mixture effect, indicating that relatively small increases in circulating Cu may have measurable effects on tumor-related metabolic pathways. Increased copper levels have been observed in many cancers such as lung, breast, prostate, stomach, thyroid, gallbladder cancer, and higher

Cu concentrations in blood and tissues are linked with tumour growth.^{17,20,49,51} The Central Water Commission (CWC) and Central Pollution Control Board (CPCB) have reported abnormal Cu levels in Indian rivers falling beyond the permissible limit [50 ppb/($\mu\text{g/L}$)], which could cause chronic environmental exposure through drinking water and other sources.⁵²

Cadmium also showed a robust association⁵³, consistent with its long biological half-life⁵⁴ (10-30 years), bioaccumulation, and ability to induce oxidative stress and inflammatory pathways in epithelial tissues.⁵³ Cd compounds (Cd chloride, Cd sulfate, Cd oxide) are known Class 1 carcinogens associated with cancers of the prostate, adrenal gland, testis and lung⁵⁵. While smoking remains the most prominent source of Cd, environmental and occupational exposure tends to be frequently ignored. According to the 2025 report of the CPCB, a total of 296 polluted river stretches (PRS) showed higher concentrations of Cd along with Cr, As and Pb specifically in the northern pockets of India.⁵⁶ Of these, the Ganga riverine system, known to sustain livelihoods across its belt, is known to have undergone significant deterioration due to numerous tanneries persisting chiefly in Kanpur, Varanasi and Patna.⁵⁷ Due to its long ecological persistence, Cd is known to bioaccumulate within the aquatic systems⁵⁸, thus navigating within the food chain.

Manganese also showed a strong and consistent association across models, suggesting a potential role in oxidative stress pathways and mitochondrial function, although its contribution to carcinogenesis remains less well characterized. However, Kumar et al. reported Mn contamination in handpump water and elevated blood Mn levels within cancer patients, specifically in Bihar, showing a significant geo-spatial distribution in the middle gangetic plain region.⁵⁹

Divalent Metal Transporter 1 (DMT1) is a transmembrane protein needed for the absorption of divalent ions in the cells, especially Fe^{2+} and Mn^{2+} , but inadvertently absorbs Cd^{2+} .⁶⁰ Chronic exposure to Cd combined with an iron deficiency upregulates the DMT1 expression, concurrently accumulating Cd and Mn within the cells.⁶⁰

The inverse association for Se is consistent with its role in antioxidant defense, particularly in the context of toxic metal exposure, which can induce oxidative stress and inflammation⁶¹. For other metals such as U and Ni, the observed associations were more complex and varied across models, indicating that their role within the mixture may depend on exposure context, co-occurring metals, or model specification.

However, the amount of heavy metal contamination persists. There is a dire need to maintain the permissible metal concentrations as laid down by the BIS^{62,63}, and stringent measures need to be maintained in the long term to reduce the metal exposure.

The imbalance in gallstone prevalence is consistent with the established role of gallstones as a major risk factor for GBC and with prior evidence linking GSD to altered hepatobiliary metal profiles^{20,21,23}, indicating potential overlap between metal exposure, GSD, and carcinogenesis.

Stratified analysis of metals across sex was carried out to assess the strength of association, as the risk of GBC is two to six times higher in females compared to males. WQS and Qgcomp regression analysis showed a similar trend with increased odds of developing GBC in females compared to males.

Cr, Cd and Mn showed a similar direction of association, with a disproportionate positive effect observed in females. Upon stratifying across the birth residential risk zones, Mn showed an increased odds of association in low-risk regions compared to high-risk regions. This could potentially involve the role of dietary, environmental or occupational exposure to elevated levels of Mn.

Cu showed a significant magnitude of association in males and among tobacco users. The direction of this association supports the role of Cu accumulation in tobacco leaves,⁶⁴ whereby Cu^{2+} ions promote the formation of tobacco specific nitrosamines (TSNA's).⁶⁵ The stratified analysis needs to be interpreted cautiously due to the relatively small sample size, which may have reduced statistical power, as the study was not originally designed for a detailed stratified analysis.

This study has several strengths. It is the largest case-control study of metals and GBC to date in India, with sufficient power to detect non-linear associations and evaluate mixture effects. In addition, simultaneous processing of cases and controls minimized measurement bias along with strict quality control checkpoints.

We also acknowledge a few limitations of this study. The use of serum may not fully capture long-term exposure to metals with short half-lives or limited serum distribution (e.g., Pb). In addition, as a case-control study with post-diagnosis sampling, reverse causation cannot be excluded, and alterations in

metal profiles may partly reflect disease-related metabolic changes rather than causal exposure pathways. Future studies should incorporate appropriate biospecimens such as urine, whole blood, or keratin-based matrices. In addition, the lack of As speciation limits the interpretation of its role, as different chemical forms have distinct toxicological properties. Future studies should integrate longitudinal designs and mechanistic biomarkers to better resolve causal pathways and temporal relationships.

Conclusion

Our study concludes that metals indeed play a significant carcinogenic role in the development of GBC, either showing a linear or a non-linear dose-response trend. Since a majority of metals function as a cluster, identification of the exposure source can be carried out by measuring the metal concentrations in soil or water samples of the high-risk regions, and then comparing them with the permissible limits. This would allow a primary prevention step in addressing the GBC risk in India by addressing environmental contamination and protecting human health.

Abbreviations

- CCE
Centre for Cancer Epidemiology
- CI
Confidence interval
- CV
Coefficient of variation
- GBC
Gallbladder Cancer
- DMGs
Disease Management Groups
- ICPMS
Inductively Coupled Plasma Mass Spectrometry
- MDL
Method detection limit
- OR
Odds ratio
- qqcomp
Quantile g-computation
- RCS
Restricted cubic spline
- SD
Standard deviation
- TMH

Declarations

Competing Interests

The authors declare no competing interests.

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Author Contribution

SM and RD conceptualised, designed and directed the study. SS carried out participant enrolment and questionnaire data acquisition. AM carried out epidemiological data cleaning, curation and management. KS standardized & validated the method, monitored the quality control and troubleshooting steps of the metal analysis. MDC, PS, KJ, SK, and KB documented and implemented the guidelines for multi-element analysis. MDC, PS, KJ, SK, KB, and RG supervised the laboratory activities and carried out the pre-processing of metal data. KK carried out pre-statistical data compilation and curation. OP, RG, and SP implemented the statistical analysis. SM, RD, KS, MDC, PS, KJ, SK, KB, and PC were involved in the interpretation of the results. MDC, KS, OP, SP, RG, PS, KJ, SK, KB, GSG, DA, AM, KK, VO, SS, PC, SG, RD, and SM documented and reviewed the manuscript. All authors provided critical feedback and helped shape the research analysis and manuscript. All authors read and approved the final version of this manuscript.

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Data Availability

Due to ethical limitations, we are unable to share the raw concentration and intensities data files for the measured metals. The respective datafiles and other essential information would be available upon a request addressed to the corresponding author, Sharayu Mhatre (smhatre@actrec.gov.in) and the senior author, Rajesh Dikshit (dixr24@hotmail.com).

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Tables

Tables are available in the Supplementary Files section.

Figures

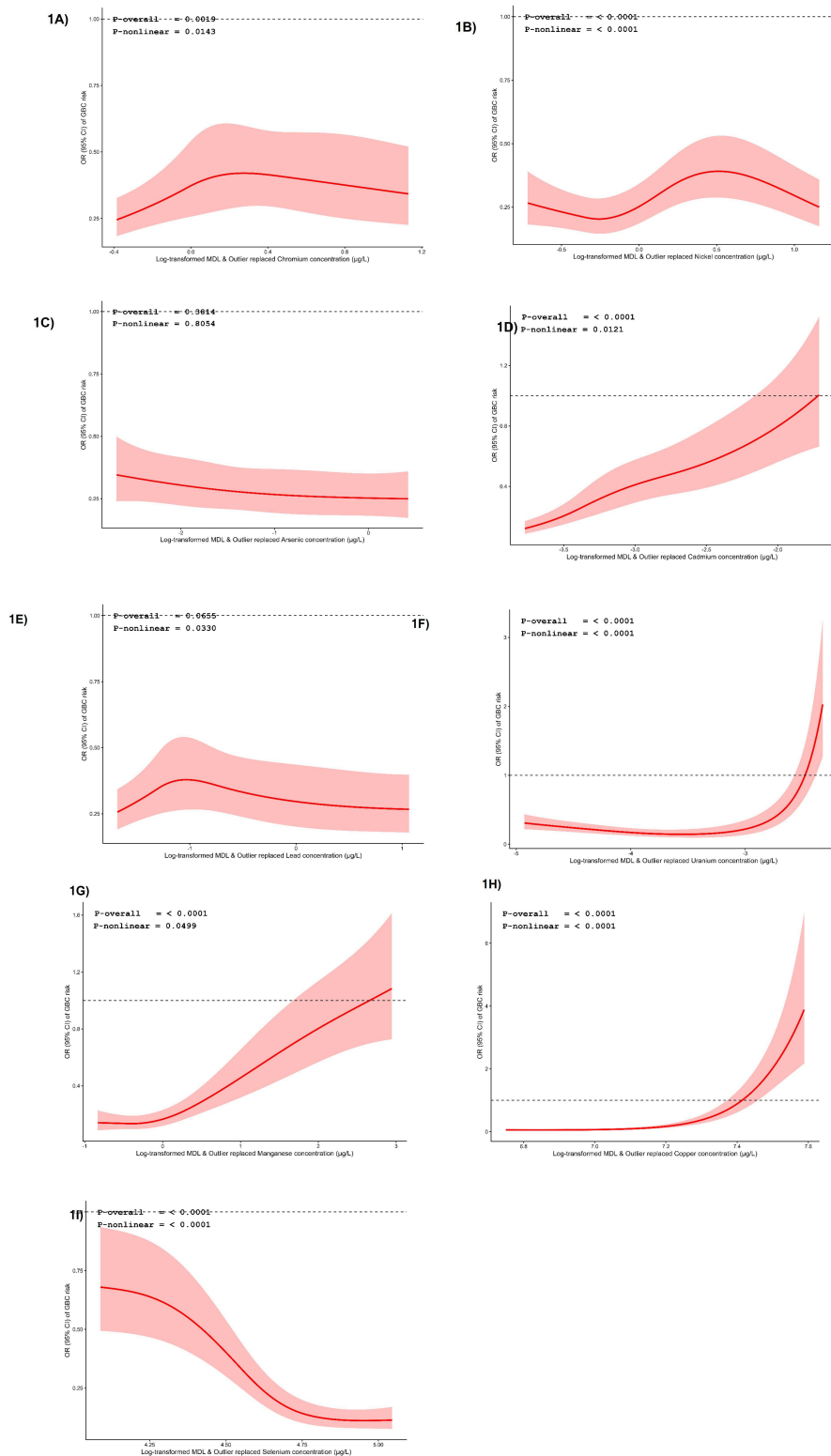


Figure 1

Restricted Cubic Spline Curves for the Association of Serum Metals with GBC Risk

The restricted cubic spline for the relationships between serum metal concentrations and the gallbladder (GBC) risk. The ORs (red lines) and 95% confidence intervals (red range) were calculated based on the

restricted cubic spline models for log-transformed metal concentrations ($\mu\text{g/L}$) after MDL adjustment ($\text{MDL}/\sqrt{2}$) and outlier replacement. The dashed horizontal line ($\text{OR} = 1.0$) represents the reference level.

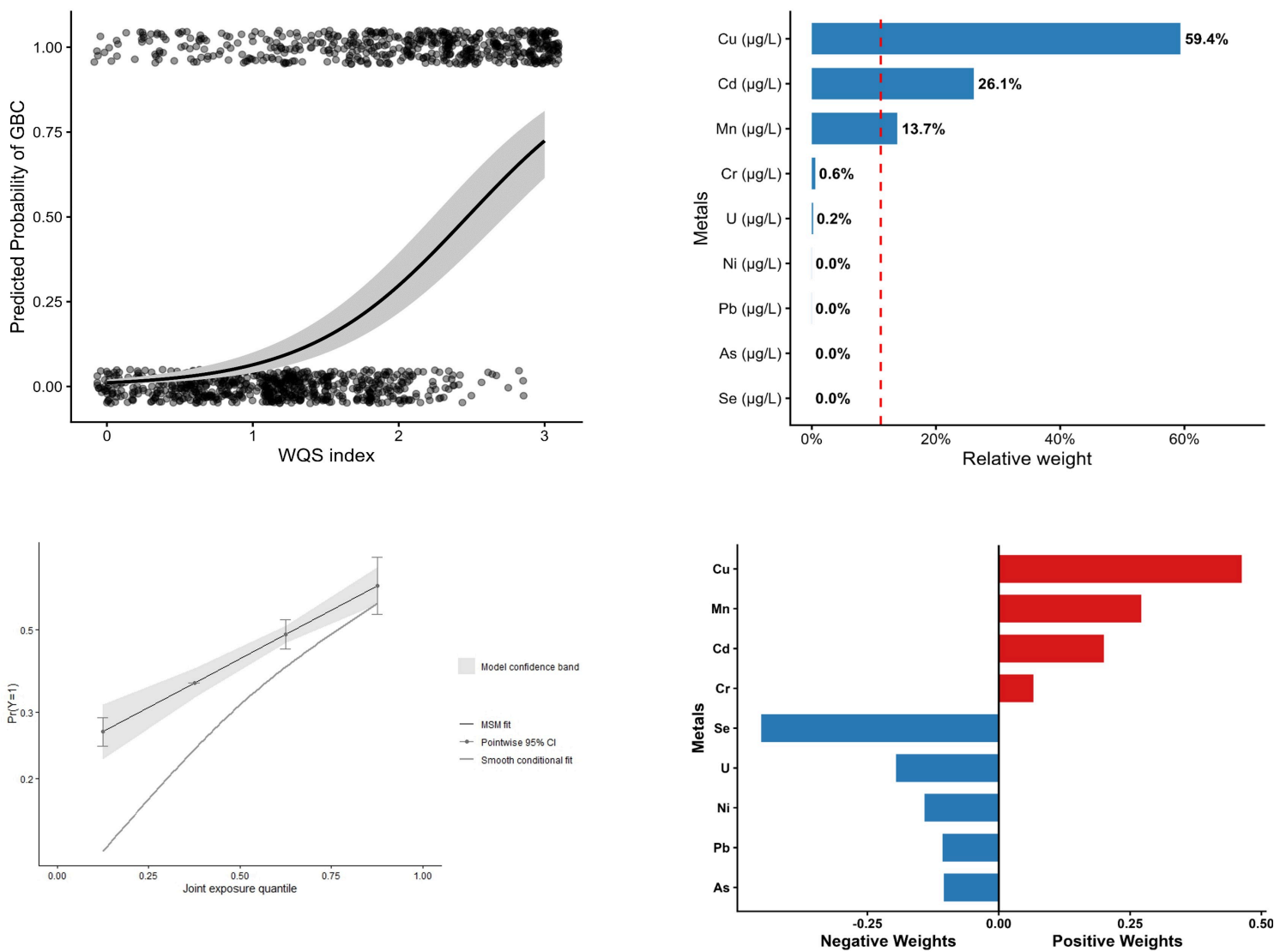


Figure 2

2A: Association Between Metal Mixture Exposure and Gallbladder Cancer Risk Estimated by WQS Regression

2A: Association between metal mixture exposure and gallbladder cancer (GBC) risk estimated using weighted quantile sum (WQS) regression. The WQS index represents the combined exposure to multiple metals. The solid black line shows the predicted probability of GBC across increasing WQS index values, while the grey shaded area represents the 95% confidence interval.

Jittered points indicate individual observations (0 = control, 1 = case), illustrating the distribution of participants across exposure levels. An increasing trend in the curve suggests a positive association between metal mixture exposure and GBC risk.

2B: Relative Contribution of Metals to Gallbladder Cancer Risk Estimated by WQS Regression

Figure 2B: Relative weights of individual metals contributing to gallbladder cancer (GBC) risk estimated using weighted quantile sum (WQS) regression. Each bar represents the proportional contribution (weight) of a specific metal to the overall mixture effect. The red dashed line indicates the threshold for equal contribution ($1/\text{number of metals}$). Metals with weights above this threshold are considered to have a stronger influence on GBC risk.

2C: Quantile G-Computation

Figure 2C: Joint effects of multiple metal exposures on gallbladder cancer (GBC) risk were evaluated using quantile g-computation (qgcomp). The outcome (GBC_CC) was modeled as a binary variable (0 = control, 1 = case), with adjustment for age (Continuous), sex (male/female), residential risk region (Low risk region/High risk region), self-reported gallstone/s (Yes/no), tobacco use (Yes/no), mustard oil consumption (continuous), and education (<5 yrs / > 5 yrs).

The exposure mixture included nine metals (Cr, U, Mn, Ni, Cu, As, Se, Cd, and Pb), categorized into quartiles ($q = 4$). Models were fitted using a binomial framework with nonparametric bootstrapping (1,000 iterations) to estimate 95% confidence intervals.

The overall mixture effect (ψ) represents the change in log-odds of GBC per one-quartile increase in all metal exposures.

2D: Quantile-Based G-Computation Analysis

Figure 2D: A quantile-based g-computation approach was used to evaluate the joint effects of multiple metal exposures on the risk of gallbladder cancer (GBC). Metal concentrations were categorized into quartiles ($q = 4$), and models were adjusted for age, gender, gallstone status, education, birth risk zone, tobacco use, and mustard oil consumption.

The overall mixture effect (ψ) represents the change in log-odds of GBC per one-quartile increase in all metal exposures. Nonparametric bootstrapping (1,000 iterations) was used to estimate standard errors and 95% confidence intervals.

Directional weights were calculated to quantify the relative contribution of each metal to the overall mixture effect. Positive weights (red) indicate metals associated with increased GBC risk, whereas negative weights (blue) indicate inverse associations. In this analysis, **Cu, Mn, Cd, and Cr showed positive weight contributions**, suggesting an increased risk of GBC, while **Se, U, Ni, Pb, and As showed negative weight contributions**, indicating inverse associations within the mixture.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1PopulationcharacteristicsV002.06.2026Final.xlsx](#)
- [Table2ORforLogHMtertileGBCV501062026Final.xlsx](#)
- [SupplementaryInformationDocumentV223.06.2026.pdf](#)