Supplementary Information

Title

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

Short Title: Serum Metal profiles and Gallbladder Cancer Risk in India

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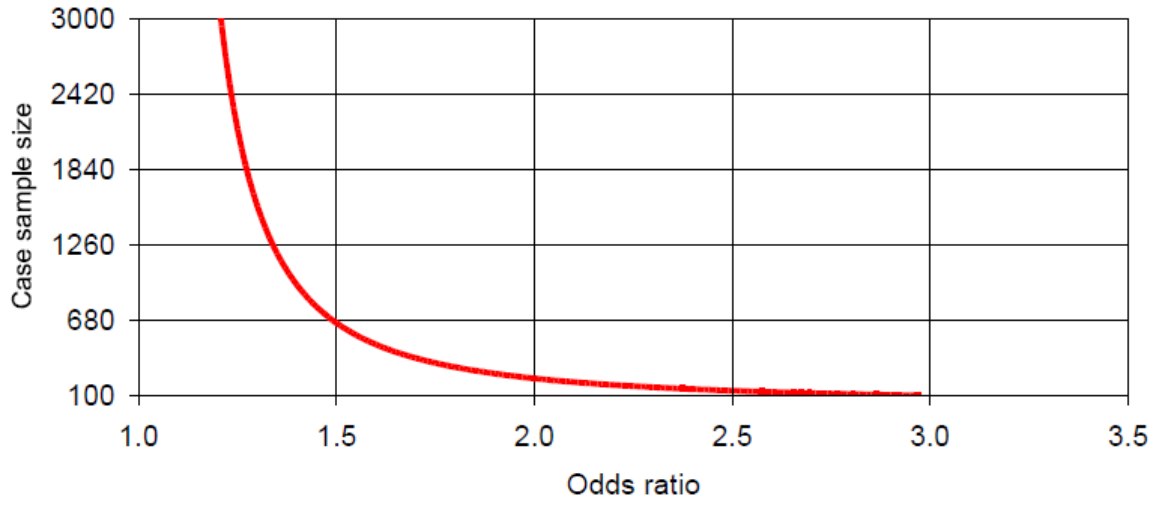
Trace metal analysis was carried out using inductively coupled plasma mass spectrometry (ICPMS). A detailed methodology regarding the sample size estimation, study design, instrument setup, chemical preparation and quality control checks are enlisted.

Sample Size Estimation:

Sample size was calculated using the Power and sample size (PS) software version 3.1.2[1] for the case-control design, assuming the prevalence of exposure in controls (p_o) of 0.5 with an expected odds ratio of 1.5. With a two-sided significance level of 0.005 along with a statistical power of 80% for 1:1 ratio of cases and controls, the estimated sample size was 658 cases and 658 controls.



Review of Literature:

Using the search query - ("Gallbladder Neoplasms"[Mesh] OR gallbladder cancer OR GBC) AND (arsenic OR cadmium OR chromium OR lead OR nickel OR "trace elements") AND (serum OR blood OR plasma OR tissue OR bile OR gallstones) within the PubMed database, research articles providing metal concentrations in biospecimens from GBC patients, particularly those employing spectrometry methods were included with no restrictions on the publication date[2–12], whereas the reviews were excluded (**supplementary table S22**)

Study Design:

In this hospital-based case-control study controls were frequency-matched to cases on the basis of age (±10 years), sex and current residential region at the time of enrolment. In order to attain optimal matching, the participants were categorized into five regions of India: North (Uttar Pradesh, Bihar, Delhi, Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Chandigarh and Uttarakhand), North-east (Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Tripura, West Bengal, Jharkhand and Orissa), West (Goa, Gujarat, Daman & Diu, Dadra & Nagar Haveli, Maharashtra), Central (Madhya Pradesh and Chhattisgarh) and South (Andhra Pradesh, Karnataka, Kerala, Lakshadweep, Andaman & Nicobar, Tamil Nadu, Telangana).

Instrumentation:

Serum trace metal concentrations from 912 GBC cases and 1140 visitor controls were measured using **Thermo Scientific™ iCAP™ TQ (triple quadrupole) ICPMS** coupled with the Thermo Scientific™ CETAC autosampler ASX-560. Data acquisition and instrument control was managed using **Qtegra™ Intelligent Scientific Data Solution™** (ISDS) software (version 2.14.5122.467, 64 bit)[13] In each analytical set, the serum sequence included 12 cases and 15 control samples, including one repeat sample, 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, (M/s Recipe, GmbH, Germany) along with a drift standard (Std 4).

Ultra-high purity gas purification panels (purity: 99.999% grade- 5.0) utilized argon gas (pressure - 0.6 MPa), helium gas (pressure - 0.15 MPa) and oxygen (pressure- 0.15 MPa) as a reaction gas (iCAP series qualification kit Manual, BRE0006846, revision B, May 24, 2017). Grade 5.0 gases indicate less than 2 ppm moisture, 1 ppm oxygen and 0.5 ppm of hydrocarbons.

The sample introduction system was equipped with a micro mist DC nebulizer, a quartz cyclonic spray chamber, Ni sampler and skimmer cones. The operating parameters of the instrument were stabilized by tuning the interface region **(supplementary table S13-S14)**.

Mn (m/z = 55), Ni (m/z = 60), Cu (m/z = 63), Cd (m/z = 111), Pb (m/z = 208), U (m/z = 238) and the internal standards gallium (Ga) (m/z = 71), rhodium (Rh) (m/z = 103) and iridium (Ir) (m/z = 193) were measured (KED) mode with 'He' gas in the collision reaction cell (CRC). Cr (m/z = 52→68 as oxygen-adduct), As (m/z = 75→91), Se (m/z = 80→96) and internal standard yttrium (Y) (m/z = 89→105) were measured in oxygen gas mode. Metal exposure pathways from the natural, dietary, anthropogenic sources[14–18] along with the optimal biospecimen for measuring the metals using ICPMS[19,20] are described in the **supplementary tables S23-S24**.

Preparation of reagents, calibration standards, internal standards and sample solution for multi-element ICPMS analysis:

The chemical preparation for calibration standards and samples were prepared using ultrapure reagents in a clean laboratory environment. Nitric acid solutions of 2% and 5% (v/v) were prepared using Milli-Q water (18.2 MΩ·cm resistivity), where 2% HNO_3 was used as the common diluent for standards, samples, certified reference materials (CRMs), and method

blanks, while 5% HNO_3 served as the rinse solution to minimize carryover between analyses **(supplementary table S4)**. Calibration working standards for chromium (Cr), manganese (Mn), nickel (Ni), copper (Cu), arsenic (As), selenium (Se), cadmium (Cd), lead (Pb), and uranium (U) were prepared by serial dilution of certified stock solutions to obtain intermediate multi-element mixtures in 2% HNO_3 **(supplementary table S5-S7)**. A multi-element internal standard solution containing gallium (Ga), yttrium (Y), rhodium (Rh), and iridium (Ir) was prepared by diluting individual 1000 ppm stock solutions to obtain a final working concentration of 1000 ppb in 2% HNO_3, and was used for signal correction, compensation of matrix effects, and monitoring instrumental drift **(supplementary table S6)**. A high working stock solution containing element concentrations ranging from 25 to 2500 ppb was prepared and further diluted to obtain a low-stock solution (0.5 mL high stock diluted to 10 mL with 2% HNO_3) **(supplementary table S8-S9)**. The diluent used for all preparations consisted of 2% HNO_3 containing 10 µg/L internal standard solution (49.5 mL 2% HNO_3 + 0.5 mL internal standard; total volume 50 mL) **(supplementary table S10)**. Seven calibration standards (STD1–STD7) were then prepared using appropriate volumes of high-stock or low-stock solutions and diluent to generate calibration curves covering the expected analytical range **(supplementary table S11)**. For sample preparation, serum samples and CRMs were diluted by adding 40 µL of sample to 1.96 mL of diluent to obtain a final volume of 2 mL (1:50 dilution) **(supplementary table S12)**. These procedures ensured consistent matrix matching, calibration linearity, and accurate quantification of trace elements during ICPMS analysis.

Calibration and Quantification:

The outlined method was validated by evaluating analytical performance parameters, including linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and Method Detection limit (MDL) by manual blank subtraction. Instrument

sensitivity was optimized during elemental tuning (iCap TQ TUNE Solution by ThermoScientific™) procedures using Kinetic energy discrimination (KED) and no gas (None) mode to reduce polyatomic interferences (background = 0, double-charge ratio ≤0.1, KED ratio ≥8, Oxide Ratio ≤0.1) and the performance report was reviewed and stored **(supplementary table S13-S14)**. A seven-point calibration curve was used for target analytes ranging from 0.01 µg/L to 100 µg/L **(supplementary table S7)**. A linear calibration curve was plotted by instrument blank subtraction and a correlation coefficient (R^2) of ≥ 0.995 was accepted for all the elements **(supplementary table S3).** The instrument detection limit (IDL) was determined based on the signal-to-noise (S/N) ratio.

Quality control and Method Validation:

Method blanks were processed flanking the serum samples to calculate the method detection limit (MDL). Quality control (QC) samples were intermittently injected throughout the analytical run to monitor and ensure the stability of the instrument response and overall system performance. CRMs including ClinCal, ClinChek Level I, ClinChek Level II, and method blanks were processed alongside the samples to validate accuracy and detect any potential contamination **(supplementary table S17)**. A drift standard (Std 4) was specifically repeated four times per sample sheet to monitor instrumental drift with an acceptable coefficient of variation of <20% and a recovery percentage of 80-120% **(supplementary table S18)**. A total of 228 serum samples were analysed in replicates for intra- assay (repeat measurements of the same samples within the same analytical run to assess precision and repeatability comparison) and 107 serum samples were processed as inter-assays (analysis of samples across different runs or days to evaluate reproducibility and method stability) **(supplementary tables S15-S16)**.

Pre-processing of serum metal data:

A preliminary processing step was carried out using the raw data exported using the Qtegra™ ISDS software. The data is then distributed into average concentrations and raw average intensities with respect to each analyte, of which the concentration values are manually subtracted from the mean of all method blanks. Mean of all method blanks was calculated primarily using box-whisker plots to eliminate potential outliers, whereas flanking method blanks with elevated congruent values between sets were used for that particular sample set. Method detection limits (MDL) of each analyte was calculated by the formula: standard deviation of all method blanks multiplied by a factor of **3.33**.[21,22] We calculated the standard deviation, coefficient of variation and recovery percentages for the CRMs, drift standard, intra-assay repeat samples and seven calibration standards.

Statistical Analysis

Logistic regression: Distinct log transformed metal concentration data was grouped into tertiles in which the lowest category included MDL/√2 replaced and lower concentration values, wherein the remaining values were categorised into the other two tertiles. The metal concentration values of the control group were used to define tertile ranges. Odds ratio (OR) and corresponding 95% confidence intervals (CI) for each metal was calculated using the metal categories by multivariate unconditional logistic regression models[23] adjusted for age, sex, birth residential risk region (High risk and low risk), self-reported gallstones (Yes/No), overall tobacco use (Yes/No), mustard oil consumption (Yes/No) and education. Overall tobacco consumption for participants reporting smoking or chewing tobacco were considered tobacco users whereas those reporting neither were never-users.[24] The P-values measured were corrected for multiple comparisons by applying the Bonferroni correction. Since we have taken nine metals into consideration, the corrected P-value was measured to be 0.0055. Therefore, the significant p-value considered for our study was ≤ 0.005.[25]

Mean serum metal-concentrations were estimated state-wise and reported only if five or more observations were recorded for an individual state. All the above-mentioned statistical analysis was performed using **Stata Version 15.0**.

Spearman Correlation matrix As our data showed a skewed distribution, a spearman correlation matrix for all the metals was plotted using the '**corrplot**' package in **R studio V. 4.5.1**[26] individually for the case and control group. Original data was utilized for computing the matrix wherein each cell denotes the correlation between two metals.

Metals showing a moderate to strong correlation (Cd, Mn and U) were subjected to a sensitivity analysis wherein Cd is adjusted for Mn & U with a similar method used in the multivariate logistic regression.

Hierarchical Cluster Analysis

Hierarchical relationships between metals was visualized using a dendrogram to identify similar metal clustering patterns between cases and controls. The correlation matrix was converted into a distance matrix (1 − correlation), and clusters were obtained using Ward's method (**Ward.D2**).[27] Clustering analysis was performed on the original data and individual metal clusters were read bottom-up. 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.

Restricted Cubic Spline (RCS)

Non-linear dose-response trends between metals was assessed using an RCS model using the '**rms**' package.[28] The model was equipped with four knots placed at the 5^{th}, 35^{th}, 65^{th}, and 95^{th} percentiles of the exposure distribution to capture complex patterns with a flexible method to identify dose response trends.

RCS was plotted on MDL outlier replaced and log transformed data. Quantile logistic regression models were applied within the RCS model to estimate odds ratios (ORs) and 95% confidence intervals (CIs) after adjusting for potential confounders with an accepted p-value of 0.0005 post Bonferroni adjustment. RCS ANOVA was applied to assess the statistical significance of the overall association and non-linearity by providing P-overall and P-nonlinear values for each metal. The p-overall value tests the linear and non-linear association between metals and GBC, whereas p-nonlinear values specifically evaluate the deviation from linearity, thus providing a non-linear trend. In the spline plots, the x-axis represents log transformed serum metal concentrations and the y-axis represents the odds ratio (OR) for GBC. The solid curve indicates the estimated dose–response relationship, whereas the dashed line represents the reference with OR=1. Values above the reference line indicates increased GBC risk, whereas values below indicate decreased risk. The pink shaded area represents the 95% confidence intervals around the spline estimates.[28]

Weighted Quantile Sum (WQS):

To study the combined effect of all metals as a mixture on GBC development, we performed WQS regression analysis on our original metal values using the '**gWQS**' package in R studio.[29] This method is suitable for estimating the combined risk of various exposures, especially when these exposures are highly correlated with each other. As our study showed a high correlation among Cd, Mn and U, with other metals showing moderate to weak correlation, this method could effectively identify and isolate individual metal effects from the metal mixture and also provide the combined metal mixture effect.

In the WQS method, the original metal data was distributed into quantiles of each metal, and then the algorithm divided the data into two halves by the ratio of 60:40, where 60% (1231) of our data was used for training the model and the calculation of weight, while 40% (821) of our data was used for validation. The training subset underwent bootstrap sampling with 1000

iterations, and at each iteration stable weights were assigned for each metal, reflecting their relative contribution to the overall mixture. At each iteration, the weighted sum was used for performing logistic regression model with maximum likelihood estimation to obtain an association between metal exposure and GBC risk. The final weights were obtained by adjusting these set of weights at each iteration, till we get a set of weights that maximize statistical association observed between metal exposure and GBC risk. The weighted quantile sum index was calculated as the sum of the product of final weight and quantile **(WQS index = ∑ (weight × quantile))**, and this WQS index value was applied to the validation data subset to determine the association with GBC (outcome) and to obtain the relative weight of every metal, which can be used to identify significant metals contributing to the metal mixture risk. The association of metal mixture was estimated using logistic regression model, and the confounders adjusted were the same as those used in the individual metal risk calculations.[29]

Quantile-based g-computation (Qgcomp):

In an attempt to address the unidirectional bias in WQS model, Qgcomp regression was applied on the original data using the '**qgcomp**' package[30] in R Studio to assess the joint effect of multiple metal co-exposures on a binary modelled outcome of GBC. After excluding the missing data, the exposure variables (metal concentrations) for 2023 participants were split into 4 quartiles to assess non-linear exposure–response ensuring comparability across metals measured on different scales. The Fit Qgcomp model was then adjusted for all the confounders mentioned in the multivariate logistic regression.

The overall metal mixture effect was estimated using the ψ (psi) parameter, representing the change in log-odds of GBC associated with a one-quartile increase in all exposures jointly, while keeping the confounders constant. These relationships were visualized using smooth exposure–response curves along with marginal structural models (MSM) with corresponding

confidence intervals (CI). To ensure the model robustness, nonparametric bootstrapping with 1,000 iterations was used to derive standard errors and 95% CI.

Additionally, directional weights were calculated from model coefficients to quantify the relative contribution of each metal to the overall mixture effect, with positive and negative weights that were normalised separately, facilitating interpretation similar to weighted quantile sum (WQS) regression.[30]

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Supplementary Table S1: Classification of visitor controls by disease management groups (DMGs) at Tata Memorial Centre (TMC)			
Sr No.	**DMGs**	**Numbers**	**Percentage (%)**
1	Preventive oncology	7	0.61
2	Pediatric solid tumours	17	1.49
3	Neuro oncology	36	3.16
4	Urology	45	3.95
5	Gynecology	47	4.12
6	Bone and soft tissue	50	4.39
7	Breast services	56	4.91
8	Paediatric hemato lymphoid	92	8.07
9	Adult haemato lymphoid	104	9.12
10	Thoracic	137	12.02
11	Gastro intestinal	201	17.63
12	Head & neck services	335	29.39
13	Missing	13	1.14
	Total Number of controls	1140	100

Supplementary Table S2: Information on the covariates used in the multivariate logistic regression (MLR) model

Sr No.	Covariate	Variables Collected	Reporting style	Criteria for the variable	Final Variable Generated
1	Age	Completed age as 'years'	Self-reported	Age as reported by the participant at the time of enrolment	Age was used in the MLR model as the continuous variable.
2	Sex	Biological sex	Self-reported	-	Sex was adjusted for in the MLR model as a dichotomous variable - males or females.
3	Smoking tobacco use	Age when product use was started, age when it was stopped, number consumed per day, number of days consumed per week	Self-reported	The participant was considered a regular smoker if they consumed a smoking tobacco product once a week for at least six months in a year.	Ever-versus never user of tobacco was finally used, where the participant could be either a consumer of smoking tobacco and/or chewing/smokeless tobacco.
4	Chewing tobacco use	Age when product use was started, age when it was stopped, number consumed per day, number of days consumed per week, site of placement	Self-reported	The participant was considered a regular chewer if they consumed a chewing/smokeless tobacco product once a week for at least six months in a year.	
5	History of gallstones	Age and the year of diagnosis, whether treatment was received and also the form of treatment, and mode of	Self-reported	-	Ever-versus-never had gallstones was adjusted for in the MLR models.

		diagnosis			
6	Education	The highest level of education received by the participant	Self-reported	-	**Education was collected as a categorical variable:** **1. Illiterate** **2. Literate** **3. Less than 5 years of schooling** **4. 5-8 years of schooling** **5. High school** **6. College graduation or more**
					Educational categories were divided into two categories that were used in the MLR model: **1. Less than 5 years of schooling (points 1-3 above)** **2. More than or equal to 5 years of schooling (points 4-6)**
7	Body Mass Index (BMI)	Height, Weight	Measured by trained interviewer	Each measurement was taken twice and the average was taken as the final measure	**Using BMI formula of Weight in kg/(Height in metres)^2**, values were categorised as per international standards: **Severely underweight:** <16·5 **Underweight:** 16·5-18·5 **Normal**: 18·5-24·9 **Overweight**: 24·9-30·0 **Obese**: >30·0

8	Birth Risk Zone	City/Village, District, State of birth	Self-reported	The place where the participant was born in.	**States were classified as zones:** **North -** Uttar Pradesh, Bihar, Delhi, Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Chandigarh, & Uttarakhand **North-east** - Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Tripura, West Bengal, Jharkhand, and Orissa **South** - Andhra Pradesh, Karnataka, Kerala, Lakshadweep, Andaman and Nicobar, Tamil Nadu, Telangana **West -** Goa, Gujarat, Daman and Diu, Dadra and Nagar Haveli, Maharashtra **Central** - Madhya Pradesh and Chhattisgarh **Zones were then classified as high and low risk zones:** **High risk -** North and North-eastern states **Low-risk -** South, West, and Central states
9	Current Residential Region	City & Village, District, State, Pin code, urban/rural status	Self-reported	Identified as the place the participant was staying at the time of enrolment	Zone classification was the same as that for birth regions.
10	Mustard Oil Consumption	Information on whether they consumed it or not (Yes/No), Family consumption quantity (Kg/Litre), the years of consumption	Self-reported	This information was collected as practised in the past one year	Ever-versus-never use of mustard oil was finally used in the multivariate logistic regression model.

Supplementary Table S3: Performance evaluation demonstrating linearity (R^2) of multi-element calibration curve (STD1 - STD7) in ICPMS Analysis

Sr No.	Analytes	Expected value*	Observed Mean Value#
1	Chromium (Cr)	≥0.995	0.999
2	Manganese (Mn)	≥0.995	0.999
3	Nickel (Ni)	≥0.995	0.999
4	Copper (Cu)	≥0.995	0.999
5	Arsenic (As)	≥0.995	0.999
6	Selenium (Se)	≥0.995	0.999
7	Cadmium (Cd)	≥0.995	0.999
8	Lead (Pb)	≥0.995	0.999
9	Uranium (U)	≥0.995	0.999

*Expected value: The minimum expected R^2 value for estimating the linearity, as per the standardized methodology.
#Observed Mean Value: The average R^2 value observed from the calibration standards (STD1–STD7) across all batches (n = 76) for each analyte of interest. Each batch consisted of 27 unique samples (12 cases and 15 controls)

Supplementary Table S4: Preparation of nitric acid solution (HNO_3) of varying concentrations for ICPMS sample processing and instrument maintenance

Sr No.	Volume HNO_3 (ml)	Milli-Q (ml)	Final concentration	Required volume (ml)	Purpose
1	4	196	2%	200	Used for diluent and blank preparation
2	50	1000	5%	1000	Rinsing solution for the sample line wash between samples

Abbreviation: ml, milliliter

Nitric acid (HNO_3) solutions were prepared using double distilled (Milli-Q, Resistivity – 18.2 MΩ·cm) water to prepare a common matrix for calibration standards, samples, certified reference material (CRMs) and method blanks.

Supplementary Table S5: Preparation of multi-element calibration standard solutions by serial dilution of 1000 ppm stock standards to 1000 ppb working concentrations in 2% HNO_3

Sr No.	Stock Elements	Cr	Mn	Ni	Cu	As	Se	Cd	Pb	U
1	Mother Stock concentration (ppm)[#]	1000	1000	1000	1000000	1000	1000	1000	1000	1000
2	Volume from Mother stock (µl)	10	10	10	25	10	100	10	10	10
3	Volume of 2% HNO_3 (µl)	9990	9990	9990	-	9990	9900	9990	9990	9990
4	Total volume (µl)	10000	10000	10000	[Added directly to High Stock[*]]	10000	10000	10000	10000	10000

Abbreviation: ppb, parts per billion; ppm, parts per million; µl, microliters; Cr, chromium; Mn, manganese; Ni, nickel; Cu, copper; As, arsenic; Se, selenium; Cd, cadmium; Pb, lead; U, uranium; HNO3, Nitric acid.

[#] Stock solution of metals with concentrations in ppm, provided by the manufacturer.

*High stock: Working multielement high concentration stock solution to prepare calibration standards.

Individual element stock solutions of all metals were diluted from their respective 1000 ppm mother stocks (except Se and Cu) to achieve a final concentration of 1000 ppb in 2% ultrapure nitric acid (HNO_3). An intermediate stock of 10000 ppb is prepared for Se whereas Cu is directly added to the high stock.

This mixed multi-element working standard was used for instrument calibration, linearity verification, and quantification of analytes during ICPMS analysis.

Supplementary Table S6: Preparation of multi-element internal standard (ISTD) solution by serial dilution from 1000 ppm stock standards to 1000 ppb working concentration in 2% HNO_3

Sr No.	Stock Elements	Ga	Y	Rh	Ir
1	Mother Stock concentration (ppm)[#]	1000	1000	1000	1000
2	Volume from Mother stock (µl)	50	50	50	50
3	Volume of 2% HNO_3 (µl)	49800			
3	Total volume (µl)	50000			

Abbreviation: ppb, parts per billion; ppm, parts per million; µl, microliters; Ga, gallium; Y, yttrium; Rh, rhodium; and Ir, iridium; HNO_3, Nitric acid.

[#] Stock solution of metals with concentrations in ppm, provided by the manufacturer.

Individual ISTD stock metal concentrations (1000 ppm) were diluted to obtain a working concentration of 1000 ppb in 50 mL of total volume. This ISTD mix was used for signal correction, matrix effect compensation, and instrumental drift adjustment during ICPMS analysis.

Supplementary Table S7: Calibration standard concentrations of metals used for ICPMS analysis

STANDARD (ppb)	Cr	Mn	Ni	Cu	Se	As	Cd	Pb	U
STD 1	0.06	0.06	0.06	1	0.2	0.02	0.01	0.02	0.01
STD 2	0.15	0.15	0.15	2.5	0.5	0.05	0.025	0.05	0.025
STD 3	0.3	0.3	0.3	5	1	0.1	0.05	0.1	0.05
STD 4	0.6	0.6	0.6	10	2	0.2	0.1	0.2	0.1
STD 5	1.5	1.5	1.5	25	5	0.5	0.25	0.5	0.25
STD 6	3	3	3	50	10	1	0.5	1	0.5
STD 7	6	6	6	100	20	2	1	2	1

Abbreviation: ppb, parts per billion; Cr, chromium; Mn, manganese; Ni, nickel; Cu, copper; As, arsenic; Se, selenium; Cd, cadmium; Pb, lead; U, uranium.

A series of seven multi-element calibration standards (STD1–STD7) were prepared from working high stock and low stock solutions to establish calibration curves for all metals.

The concentrations were selected to cover the expected analytical range of the samples, ensuring linearity, precision, and accuracy in quantitative ICPMS determination.

Supplementary Table S8: Preparation of multi-element high working stock solution (1000 ppb to intermediate concentrations) in 2% HNO_3 for ICPMS calibration curve

Sr No.	Stock	Cr	Mn	Ni	Cu	As	Se	Cd	Pb	U		
1	Stock concentration (ppb)*	1000	1000	1000	1000000	1000	10000	1000	1000	1000		
2	Required concentration (ppb)#	150	150	150	2500	50	500	25	50	25		
3	Volume from stock (µl)	1500	1500	1500	25	500	500	250	500	250		
4	Volume of 2% HNO_3 (µl)	3475										
5	Total volume (µl)	10000										

Abbreviation: ppb, parts per billion; µl, microliters; Cr, chromium; Mn, manganese; Ni, nickel; Cu, copper; As, arsenic; Se, selenium; Cd, cadmium; Pb, lead; U, uranium.

* Stock concentration used here metal concentration in ppb prepared by serial dilution of 1000 ppm mother stock standards.
# Required concentrations for higher concentration of calibration standards.

Working stocks of individual metals were diluted from their respective 1000 ppb solutions to prepare a high stock solution.
Aliquots of the required volumes of each element were combined and brought to a final volume of 10 mL with 2% ultrapure nitric acid (HNO_3) to achieve target concentrations ranging from 25 to 2500 ppb, depending on the element.
This high working stock served as the intermediate standard for subsequent preparation of calibration standards (STD4–STD7) used in quantitative ICPMS analysis.

Supplementary Table S9: Preparation of low-stock[#] multi-element working standard by dilution of high-stock solution in 2% Nitric acid (HNO_3).

Component	Volume (ml)
High-stock multielement standard[*]	0.5
2% Nitric acid (HNO_3, freshly prepared)	9.5

Abbreviation: ml, milliliter.

[*]High-stock multielement standard served as the intermediate standard for subsequent preparation of the low stock for standards (STD1–STD3) used in ICPMS analysis.

[#]A low-stock multi-element working standard was prepared by diluting 0.5 mL of the high-stock multi-element standard to a final volume of 10 mL using freshly prepared 2% ultrapure HNO_3. This low-stock solution served as the secondary working standard for preparing calibration levels (STD1–STD3) used in quantitative ICPMS analysis.

Supplementary Table S10: Preparation of Diluent Solution* for ICPMS Analysis		
2% Nitric acid (ml)	**Internal Std Mix (ml)**	**Total Vol. (ml)**
49.5	0.5	50

Abbreviation: ml, milliliters.

*This table summarizes the composition and volumes used to prepare the diluent solution for preparation of samples and standards in ICPMS analysis. The diluent consists of 2% nitric acid (HNO_3) to maintain common matrix, and internal standards to correct for instrumental drift and matrix effects.

Supplementary Table S11: Preparation of Standard Dilutions[α] for ICPMS Calibration Linearity Plot			
STANDARDS	**HIGH STOCK (µl)[*]**	**LOW STOCK(µl)[#]**	**DILUENT(µl)[β]**
STD 1	-	80	9920
STD 2	-	200	9800
STD 3	-	400	9600
STD 4	40	-	9960
STD 5	100	-	9900
STD 6	200	-	9800
STD 7	400	-	9600

Abbreviation: µl, microliters.

[α] This table provides the volumes used to prepare a series of standard solutions for obtaining the linear regression in ICPMS analysis. The standards are prepared by diluting either high-stock or low-stock multielement solutions with the diluent to achieve the desired concentrations.

[*] HIGH STOCK (µL): Volume of high-concentration stock solution added to the standard. A dash (–) indicates that no high-stock solution was used for that standard.

[#] LOW STOCK (µL): Volume of low-concentration stock solution added. A dash (–) indicates that no low-stock solution was used for that standard.

[β] DILUENT (µL): Volume of diluent (2% HNO_3) used to make up the final standard volume.

Supplementary Table S12: Preparation of Samples[*] (Serum samples and Certified Reference Materials) for ICPMS Analysis			
Sr. No.	Sample Volume (µl)[α]	Diluent (µl)[β]	Total volume (µl)
1	40	1960	2000

Abbreviation: µl, microliters.

[*]This table summarizes the preparation of samples, certified reference materials (CRMs) and method blanks, prior to ICPMS analysis. The samples are diluted with an appropriate diluent ensuring accurate measurement and comparability across all samples.
[α] Sample Volume (µL): Volume of the serum sample taken for 50-folds dilution.
[β] Diluent (µl): Volume of 2% HNO_3 added to the samples to achieve the desired concentration and matrix.

Supplementary Table S13: Summary of '43' instrument tune parameters, standard and observed operating ranges used for instrument calibration and performance verification				
Sr No.	**Parameters**	**Standard Tune Values [a]**	**Observed Ranges [b]**	**Purpose**
1	Additional Gas Flow 1	0	=0	Provides a controlled supply of Argon gas to maintain plasma robustness and aerosol transport.
2	Additional Gas Flow 2	52	=52	
3	Q1 Entry Lens	-55.61	(-49.5) to (-55.61)	Electrostatic lens to allow ion transport from interface region into Q1
4	Angular Deflection	-250	=-250	An electrostatic deflection to maintain a central ion trajectory.
5	Deflection Entry Lens	-35	=-35	Assists angular deflection unit in maintaining ion beam prior to Q1 entry.
6	Extraction Lens 1 Polarity	0	=0	Set of voltages applied to extract ions from the plasma with ideal polarity and energy for transmission.
7	Extraction Lens 1 Negative	0	=0	
8	Extraction Lens 1 Positive	0	=0	
9	Spray Chamber Temperature (°C)	2.07	=2.07	Helps in controlling aerosol condensation and solvent load on the plasma, thus improving stability by controlling oxide formation.
10	Peristaltic Pump Speed (rpm)	40	=40	Controls the rate of sample uptake into the sample introduction system
11	Cool Flow (L/min)	14	=14	Primary argon flow that maintains the outer plasma, maintaining temperature and stability.
12	Sampling Depth (mm)	9	=9	Distance between the load coil and sampler cone orifice impacting sensitivity and tolerance of matrix.
13	Plasma Power (W)	1550	= 1550	Radio Frequency (RF) power to sustain and maintain plasma for ideal ionization.
14	Auxiliary Flow (L/min)	0.8	= 0.8	Fine tunes plasma position relative to the sampling cone
15	Nebulizer Flow (L/min)	0.55	0.55-0.6	Controls aerosol formation and allows efficient

				transportation of the samples.
16	Torch Horizontal Position (mm)	-0.72	(-0.72) to (-1.34)	Aligns the plasma with the sampling cone axis for optimum transmission and stable signal.
17	Torch Vertical Position (mm)	-0.9	(-0.90) to (-1.15)	
18	Extraction Lens 2 (V)	-161.8	(-154) to (-161.8)	Secondary extraction voltage for improving ion focussing and reducing neutral species before Q1
19	Q1 Focus Lens (V)	1.06	= 1.06	Refines ion focussing into Q1 thus maintaining mass identification and optimum resolution.
20	CR Bias (V)	-20	(-20) to (-21)	Sets energy barrier for the ions entering the CRC thus maintaining ion kinetic energy.
21	CR Exit Lens (V)	-40	= -40	Extracts ions from the CRC with ideal energy to transmit into Q2
22	Focus Lens (V)	-8.5	= (-8.5) to (-9.0)	Adjusts the ion beam after the ions leave the CRC thus improving ion transmission.
23	D1 Lens (V)	-350	= -350	Deflector Lens that guides ions from the CRC to the detector path
24	D2 Lens (V)	-163.63	= -163.63	Secondary deflector that stabilizes ion path and reduces background noise.
25	CR Entry Lens (V)	-100	=-100	Controls ion injection energy into the CRC, which is critical in controlling sample scattering and improving ion selectivity.
26	Quad Entry Lens (V)	-60	(-58) to (-60)	Focuses ions to the Q2 for mass calibration.
27	Pole Bias (V)	-18	=-18	Sets DC offset on the quadrupole rods, thus improving ion transmission and reduces mass bias.
28	Pole Bias Q1	0	=0	
29	CR1 Flow (ml/min)	4.175	3.70 to 4.175	Collision/reaction gas flow rate which tunes KED
30	CR2 Flow (ml/min)	0	=0	Additional CRC gas channels for multi gas modes. It is zero when inactive.
31	CR3 Flow (ml/min)	0	=0	
32	CR4 Flow (ml/min)	0	=0	
33	CR RF High Mass Amplitude Factor	60	=60	RF voltage parameters to confine high mass ions affecting transmission of heavy elements.

34	CR RF High Mass Amplitude Offset (V)	0	=0	
35	CR RF High Mass Amplitude Exponent (V)	1	=1	
36	CR RF Low Mass Amplitude Factor	60	=60	RF voltage parameters to confine low mass ions affecting transmission of heavy elements.
37	CR RF Low Mass Amplitude Offset (V)	-20	=0 to -20	
38	CR RF Low Mass Amplitude Exponent (V)	1	=1	
39	CR parameter Mass Switch Point	300	=300	Defines transition point for high mass and low mass analytes.
40	Q1 SQ Mode RF Dac Factor	450	=450	Improves resolution, mass calibration and ion stability by controlling the Q1 amplitude and offset
41	Q1 SQ Mode RF Dac Offset	-1047.5	=-1047.5	
42	Q1 SQ mode parameter b	0.65	=0.65	
43	Dry Pump Speed	77	=77	Controls the vacuum for maintaining optimum pressure in the interface region to improve ion transmission.

[a] Standard Tune Values: Predefined setpoints adjusted during the instrument's standard tuning routine to ensure optimum calibration, sensitivity, and plasma stability prior to all the analytical runs.

[b] Observed Ranges: Observed working range during instrument calibration and performance verification. Minor deviations within these ranges indicate normal instrument variability and stability.

The mentioned values span all the batches (n = 76) for each analyte of interest. Each batch consisted of 27 unique samples (12 cases and 15 controls)

Supplementary Table S14: Instrument performance check, gas check and tuning parameters indicating mass calibration accuracy, sensitivity, and vacuum integrity								
Sr. No	**Quality Control checks**	**Analytes of Interest**	**Expected values**	**Conditional limits**	**Observed values**	**Result**	**Rationale for QC checks**	**Purpose**
1	Mass Calibration Test	—	—	—	Channels: 75, Dwell: 0.04, Measure Width: 1.5, Point Spacing: 0.02, Sweeps: 5	Passed	All passed as the analyte masses are within acceptable range of their respective atomic masses Mass calibration is 'Failed' if the four analytes are not estimated at specific atomic masses	Ensures that the instrument measures the optimum atomic mass of the mentioned analytes in the Tune solution. A 'Passed result' confirms the accuracy of the mass calibration.
		Li	7	—	7.0123	Passed		
		Co	59	—	58.9215	Passed		
		In	115	—	114.8895	Passed		
		Bi	209	—	208.985	Passed		
2	Sensitivity & Stability Test	—	—	—	Runs: 30, Sweeps: 6	Passed	All the analytes are measured in counts per second (CPS) which is the signal intensity produced by the number of analyte ions hitting the detector in one second. Oxide Ratio and Double charged ions cause overlaps and spectral interferences, thus need to be controlled.	Identifies signal intensity (sensitivity) and reproducibility (specificity) of the respective analytes in the Tune solution.
		KED ratio		>8.00	11	passes		
2a	Sensitivity	Li	7	> 8,000 CPS	42,527 CPS	Passed		
		Co	59	> 16,000 CPS	95,838 CPS	Passed		
		U	238	> 35,000 CPS	301,594 CPS	Passed		

		Ce.16O / Ce	140/140	< 0.1	0.0123	Passed	A 'Failed' result would indicate any of these parameters not fulfilling the set conditional limits	
		Ce++ / Ce	140/140	< 0.1	0.033	Passed		
		In	115	> 38,000 CPS	213,465 CPS	Passed		
2b	Stability	Li	7	< 5%	1.60%	Passed	Stability of the analytes is measured in % RSD. The set relative standard deviation should be below 5%. A 'Failed' result is when the % RSD surpasses the limit of 5%	
		Co	59	< 5%	0.90%	Passed		
		U	238	< 5%	1.20%	Passed		
		In	115	< 5%	1.20%	Passed		
3	Vacuum Check	Analyzer Pressure	—	—	3.445×10^{-7}	Vacuum OK	The vacuum is adequate in the interface region. A 'Failed' result would indicate leaks or blockages in the interface region.	Ideal vacuum pressure in the interface region for optimum ion transmission
		Interface Pressure	—	—	1.85E+00	Passed		
4	Gas Channel (Sensitivity)	Co/Cl.O	59/35.16	>10	37.3	Passed	Based upon the conditional limits of the KED ratio and Cobalt values, the CRC stability is checked. A 'Failed' result would indicate impurities in the CRC or helium gas line.	To check proper operation of the Collision/reaction cell for sensitivity and stability
		Co	59	>20,000.0 CPS	27,542.0 CPS	Passed		
	Gas Channel (Stability)	Co	59	0	1.3%	Passed		

Abbreviations: QC, Quality Control; KED, Kinetic Energy Discrimination; CPS, counts per Second; RSD, Relative Standard Deviation; CRC, Collision Reaction Cell; Li, Lithium; Co, Cobalt; In, Indium; Bi, Bismuth; U, Uranium; CeO^+/Ce^+, Cerium Oxide Ratio; Ce^{2+}/Ce^+, Cerium Doubly Charged Ratio; Co^+/ClO^+, Cobalt to Chlorine Oxide Ratio

Supplementary Table S15: Analytical precision and reliability of Intra-assay measurements based on coefficient of variation (CV) and Intraclass correlation coefficient (ICC) in ICPMS analysis		
Elements	**Observed Intra-assay CV (%) [a]**	**Intraclass Correlation Coefficient (ICC) [b]**
Non-essential Metals		
Chromium	44%	0.10
Nickel	15%	0.69
Arsenic	12%	1.00
Cadmium	22%	0.93
Lead	22%	0.59
Uranium	7%	0.98
Essential Metals		
Manganese	11%	0.71
Copper	1%	0.99
Selenium	2%	0.98

Abbreviations: CV, Coefficient of Variation; ICC, Intraclass Correlation Coefficient.
The acceptable intra-assay-coefficient of variation (CV%) for each analyte was standardized at ≤20%.
a Observed Intra-assay coefficient of variation (CV%)
The intra-assay CV measures the precision and variability of repeated measurements within a single analytical run or batch. CV was calculated using the formula:
Standard Deviation/ Mean×100
Where:
SD = standard deviation of replicate measurements within the same batch
Mean = average of the replicate measurements within the same batch
Lower CV values indicates higher precision. Values above the acceptable threshold (>20%) suggest higher variability.
b Intraclass correlation coefficient (ICC)
ICC assesses the reliability of repeated measurements across different runs, batches, or raters. It evaluates how strongly measurements within the same group (batch) resemble each other relative to the total variation. In this study, ICC was calculated using a two-sided ANOVA without replication, as follows:
Perform two-sided ANOVA without replication across all batches (N=76):

Supplementary Table S16: Analytical precision and reliability of Inter-assay measurements-based coefficient of variation (CV) and intraclass correlation coefficient (ICC) in ICPMS analysis		
Elements	**Observed Inter-assay CV (%) [a]**	**Intraclass Correlation Coefficient (ICC) [b]**
Non-essential Metals		
Chromium	61%	0.09
Nickel	27%	0.74
Arsenic	8%	0.96
Cadmium	35%	0.94
Lead	34%	0.93
Uranium	11%	0.88
Essential Metals		
Manganese	29%	0.95
Copper	6%	0.59
Selenium	9%	0.58

Abbreviations: CV, Coefficient of Variation; ICC, Intraclass Correlation Coefficient.

The acceptable inter-assay: coefficient of variation (CV%) for each analyte was standardized at ≤20%.

[a] Observed Inter-assay coefficient of variation (CV%)

The inter-assay CV measures the precision and variability of repeated measurements within a single analytical run or batch. CV was calculated using the formula:

Standard Deviation/ Mean×100

Where:

SD = standard deviation of replicate measurements within the same batch

Mean = average of the replicate measurements within the same batch

Lower CV values indicate higher precision. Values above the acceptable threshold (>20%) suggest higher variability.

[b] Intraclass correlation coefficient (ICC)

ICC assesses the reliability of repeated measurements across different runs, batches, or raters. It evaluates how strongly measurements within the same group (batch) resemble each other relative to the total variation. In this study, ICC was calculated using a two-sided ANOVA without replication, as follows:

Perform two-sided ANOVA without replication across a limited sample size (N=107):

Supplementary Table S17: Analytical performance evaluation using certified reference materials (CRMs) (ClinCal and ClinChek Level I & II) - summary of certified values, recovery percentage, and intra-assay coefficients of variation for ICPMS measurement of essential and non-essential metals												
Elements	**Certified Value (ClinCal) [(µg/l)/ppb] [a]**	**Observed Value (ClinCal) [(µg/l)/ppb] [b]**	**Observed Recovery % (ClinCal)[c]**	**Observed Intra-assay CV % (ClinCal)[d]**	**Certified Value (ClinChek LI) [(µg/l)/ppb] [a]**	**Observed Value (ClinChek LI) [(µg/l)/ppb] [b]**	**Observed Recovery % (ClinChek LI)[c]**	**Observed Intra-assay CV % (ClinChek LI)[d]**	**Certified Value (ClinChek LII) [(µg/l)/ppb] [a]**	**Observed Value (ClinChek LII) [(µg/l)/ppb] [b]**	**Observed Recovery % (ClinChek LII)[c]**	**Observed Intra-assay CV % (ClinChek LII)[d]**
Non-essential Metals												
Nickel	12.2	12.5	103%	4%	2.69	2.53	116%	9%	6.69	6.85	110%	6%
Arsenic	29.6	29.8	101%	4%	10.4	10.64	109%	4%	19.9	20.9	107%	4%
Cadmium	9.54	9.20	96%	4%	1.98	1.84	94%	4%	5.76	5.18	88%	4%
Essential Metals												
Manganese	12.1	12.9	106%	5%	2.66	2.713	109%	6%	6.49	5.971	94%	4%
Copper	1710	1669	98%	3%	659	655	92%	3%	1300	1261	92%	3%
Selenium	127	128.3	101%	5%	48.8	52.31	96%	5%	94.8	97.8	96%	6%

Abbreviations: CV, Coefficient of Variation; µg/l, microgram per liter; ppb, parts per billion.

CRMs, along with their repeats, were processed across all batches (n=76) to evaluate precision and reliability.

[a] Certified Value: Reference concentration of each analyte as provided by the manufacturer (Recipe)
Expected Criteria for Recovery (%): Target range for method accuracy was standardized within 80–120%. A recovery within the target range indicates accurate measurement.

[b] Observed value: Average of respective CRM concentration from all sample sheets (n=76).

[c] Observed Recovery (%): Calculated using the formula Recovery (%) = Certified value /Mean measured concentration×100. A recovery within the target range indicates accurate measurement.
Expected Criteria for CV (%): It is the threshold value for intra-assay precision; values and ≤20% is considered as an expected criteria.

[d] Observed Intra-assay CV (%): It measures the precision of repeated measurements within the same analytical run calculated as: CV (%) = Standard Deviation (SD)/Mean×100

Supplementary Table S18: Evaluation of instrument drift and analytical precision using calibration standard 4 - summary of certified values, expected and observed values of recovery percentage, intra -assay coefficients of variation (CV), and intraclass correlation coefficient (ICC)

Elements	Expected certified Value [(µg/l)/ppb] [a]	Observed value [(µg/l)/ppb] [b]	Observed Recovery % [c]	Observed Intra-assay CV (%) [d]	Intraclass Correlation Coefficient (ICC) [e]
Non-essential Metals					
Chromium	0.6	0.6	105%	6%	0.74
Nickel	0.6	0.6	104%	11%	0.96
Arsenic	0.2	0.2	99%	11%	0.74
Cadmium	0.1	0.1	97%	8%	0.70
Lead	0.2	0.2	103%	18%	0.98
Uranium	0.1	0.1	96%	4%	0.85
Essential Metals					
Manganese	0.6	0.6	103%	5%	0.92
Copper	10	10.1	101%	4%	0.79
Selenium	2	1.9	96%	16%	0.84

Abbreviations: CV, Coefficient of Variation; µg/l, microgram per liter; ppb, parts per billion.

Standard 4 was analysed four times in all batches (n=76) to estimate instrument drift.
[a] Certified Value: Calibration curve values of Std 4 for all the analytes are chosen as the certified values for each batch.

[b] Observed value: Average of std 4 concentration from all sample sheets (n=76).

Expected Criteria for Recovery (%): Target range for method accuracy was standardized within 80–120%. A recovery within the target range indicates accurate measurement.
[c] Observed Recovery (%): Calculated as Recovery (%) = Certified value/ Mean measured concentration×100

Expected Criteria for CV (%): It is the threshold value for intra-assay precision and ≤20% is considered as an acceptable criteria.
[d] Observed Intra-assay CV (%): It measures precision of repeated measurements within the same analytical run, calculated as: CV (%) = Standard Deviation (SD) / Mean×100

[e] ICC: Calculated using two-sided ANOVA without replication: $ICC=MSrow+(k-1)\cdot MSerrorMSrow-MSerror$

Supplementary Table S19: Summary Statistics of 9 Metal Analytes (Non-essential and Essential)																		
	Non-essential Metals												Essential Metals					
	Cr (ug/L)		Ni (ug/L)		As (ug/L)		Cd (ug/L)		Pb (ug/L)		U (ug/L)		Mn (ug/L)		Cu (ug/L)		Se (ug/L)	
	Case	Controls	Case	Controls	Case	Controls	Case	Controls	Case	Controls	Case	Controls	Case	Controls	Case	Controls	Case	Controls
N	912	1140	912	1140	912	1140	912	1140	912	1140	912	1140	912	1140	909	1139	912	1140
Minimum	-12.52	-12.31	-0.44	-0.27	-0.10	-0.07	-0.05	-0.05	-0.77	-0.74	0.00	0.00	-2.64	-2.30	0.28	477.61	-0.25	0.11
Percentile 10^{th}	-0.06	-0.15	0.59	0.58	0.09	0.08	0.02	0.00	0.02	-0.01	0.00	0.00	0.82	0.57	100.08	892.39	63.11	78.66
Percentile 25^{th}	0.26	0.11	0.86	0.79	0.14	0.13	0.03	0.01	0.12	0.07	0.01	0.01	1.17	0.78	1217.26	1027.70	79.00	93.55
Percentile 50^{th}	0.66	0.46	1.15	1.10	0.23	0.22	0.06	0.02	0.24	0.18	0.01	0.01	2.76	1.11	1516.43	1174.34	95.79	109.50
Percentile 75^{th}	1.33	1.03	1.61	1.57	0.38	0.41	0.10	0.05	0.47	0.41	0.08	0.01	14.39	1.68	1920.78	1316.55	111.65	125.11
Percentile 90^{th}	2.20	1.99	2.30	2.40	0.69	0.92	0.16	0.09	1.13	1.18	0.10	0.05	18.20	7.83	2340.45	1498.65	129.13	145.93
Percentile 99^{th}	8.41	7.26	5.03	16.61	3.41	6.99	0.47	0.24	11.98	11.79	0.18	0.09	27.65	20.85	2956.43	1975.50	178.65	213.81
Maximum	47.12	45.75	13.84	145.94	27.74	30.13	4.32	1.00	83.33	74.57	0.37	0.16	69.85	48.04	3267.42	2886.28	244.63	253.11
Mean	1.12	0.87	1.38	1.84	0.44	0.55	0.09	0.04	0.86	0.72	0.04	0.02	7.58	2.88	1593.99	1189.06	96.26	111.35
SD	3.13	2.78	1.05	5.76	1.30	1.52	0.17	0.05	3.86	2.95	0.04	0.02	8.16	5.11	517.25	247.52	28.67	29.96

Sum of method blanks for MDL calculation	506	542	564	564	545	555	539	539	557
Weighted Average MDL	0.961	0.690	0.068	0.032	0.263	0.003	0.613	1.357	0.684
Samples below weighted average MDL	1398	313	102	818	1172	18	189	8	8
% of Samples below weighted average MDL	68%	15%	5%	40%	57%	1%	9%	0%	0%
Limit of Quantification (LOQ)	2.887	2.073	0.204	0.096	0.789	0.010	1.840	4.076	2.053
Intra-assay CV%	44%	15%	12%	22%	22%	7%	11%	1%	2%
Permissible limit [ug/L]	1.4	0.2 -0.5	< 1	<1	< 1	1.38 (Blood)	5-25 (Serum/Blood)	635. - 1589	70 - 130

Supplementary Table S20: Gender, Birth residence risk and Tobacco consumption stratified Odds ratio (with 95% CI) calculated for separate tertiles of Log transformed Metal values measured in Serum																		
Metals	**Categories (ug/L) #**	***Sex* †1**						***Birth residential risk zones* †2**						***Any tobacco use* †3**					
		Females			**Males**			**Low risk birth Region**			**High risk birth region**			**Non-tobacco users**			**Tobacco users**		
		Case/Control	**OR (95% CI) †**	**p-value§**	**Case / Control**	**OR (95% CI) †**	**p-value§**	**Case / Control**	**OR (95% CI) †**	**p-value§**	**Case / Control**	**OR (95% CI) †**	**p-value§**	**Case / Control**	**OR (95% CI) †**	**p-value§**	**Case / Control**	**OR (95% CI) †**	**p-value§**
Chromium (Cr)	tert 1 (0.68 — 0.68)	386/677	ref		189/146	ref		96/394	ref		479/429	ref		388/645	ref		185/175	ref	
	tert 2 (—)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	tert 3 (0.96 — 7.263)	230/254	**1.57 (1.18 - 2.08)**	**≤0.005**	107/63	1.49 (0.97 - 2.28)	0.067	60/145	**2.30 (1.41 - 3.75)**	**0.001**	275/172	1.41 (1.08 - 1.85)	0.011	234/247	**1.56 (1.18 - 2.05)**	**0.001**	100/67	1.50 (0.95 - 2.36)	0.075
	P_{trend}	**0.002**			0.067			**0.001**			0.011			**0.001**			0.075		
	$P_{interaction}$	0.678						0.076						0.910					
Nickel (Ni)	tert 1 (0.488 —	176/333	ref		69/47	ref		35/175	ref		210/205	ref		170/309	ref		73/69	ref	

	0.90)																		
	tert 2 (0.90 — 1.39)	212/311	1.18 (0.85 - 1.65)	0.311	119/69	0.99 (0.58 - 1.70)	0.988	56/178	1.28 (0.68 - 2.39)	0.428	275/202	1.16 (0.85- 1.59)	0.338	229/297	1.21 (0.88 - 1.67)	0.236	101/81	1.09 (0.63 - 1.87)	0.743
	tert 3 (1.39 — 16.62)	228/287	**1.81 (1.30 - 2.50)**	**≤0.005**	108/93	0.76 (0.45 - 1.29)	0.317	65/186	1.88 (1.04 - 3.40)	0.036	269/194	1.34 (0.98 - 1.83)	0.064	223/286	1.52 (1.11 - 2.09)	0.009	111/92	1.29 (0.76 - 2.20)	0.341
	P_{trend}	**≤0.005**			0.260			0.032			0.064			0.009			0.326		
	$P_{interaction}$	**≤0.005**						0.136						0.286					
Arsenic (As)	tert 1 (0.048 — 0.165)	205/345	ref		71/35	ref		52/189	ref		224/191	ref		202/321	ref		73/56	ref	
	tert 2 (0.166 — 0.325)	227/293	0.94 (0.68 - 1.29)	0.721	107/87	0.49 (0.28 - 0.86)	0.014	42/148	0.74 (0.40 - 1.34)	0.322	291/232	0.87 (0.64 - 1.19)	0.406	237/297	0.83 (0.61 - 1.13)	0.254	95/83	0.74 (0.42 - 1.28)	0.288
	tert 3 (0.325 — 6.994)	184/293	0.86 (0.61 -	0.385	118/87	0.51 (0.29 - 0.90	0.020	62/202	0.70 (0.40 -	0.221	239/178	0.86 (0.62 -	0.381	183/274	0.73 (0.53 -	0.071	117/103	0.79 (0.46 -	0.398

			1.20)			)			1.23)			1.19)			1.02)			1.35)	
	P_{trend}	0.388			0.044			0.229			0.383			0.069			0.466		
	$P_{interaction}$	0.056						0.857						0.860					
Cadmium (Cd)	tert 1 (0.023 — 0.023)	116/567	ref		75/60	ref		30/330	ref		161/297	ref		128/516	ref		63/109	ref	
	tert 2 (0.032 — 0.0416)	62/117	**2.42 (1.50 - 3.89)**	**≤0.005**	26/16	1.33 (0.59 - 2.97)	0.486	13/58	2.10 (0.84 - 5.27)	0.111	75/75	**1.92 (1.23 - 2.99)**	**0.004**	56/101	**2.17 (1.34 - 3.52)**	**0.002**	31/31	1.72 (0.85 - 3.44)	0.125
	tert 3 (0.0417 — 0.241)	438/247	**8.95 (6.41 - 12.50)**	**≤0.005**	195/133	1.06 (0.66 - 1.70)	0.789	113/151	**7.10 (3.98 - 12.66)**	**≤0.005**	518/229	**4.12 (3.06 - 5.53)**	**≤0.005**	438/275	**6.21 (4.54 - 8.48)**	**≤0.005**	191/102	**2.43 (1.49 - 3.95)**	**≤0.005**
	P_{trend}	**≤0.005**			0.850			**≤0.005**			**≤0.005**			**≤0.005**			**≤0.005**		
	$P_{interaction}$	**≤0.005**						0.026						**≤0.005**					
Lead (Pb)	tert 1 (0.186 —	334/597	ref		148/93	ref		72/359	ref		409/331	ref		325/546	ref		154/140	ref	

	0.186)																		
	tert 2 (0.26 — 0.31)	42/55	1.39 (0.81 - 2.41)	0.229	15/15	0.64 (0.25 - 1.61)	0.350	10/32	1.48 (0.54 - 4.05)	0.438	47/38	0.99 (0.58 - 1.70)	0.990	40/56	1.09 (0.63 - 1.88)	0.743	17/14	1.27 (0.51 - 3.14)	0.597
	tert 3 (0.31 — 11.8)	240/279	1.46 (1.10 - 1.94)	0.008	133/101	0.81 (0.53 - 1.23)	0.328	74/148	**2.74 (1.65 - 4.53)**	**≤0.005**	298/232	1.03 (0.79 - 1.34)	0.814	257/290	1.29 (0.98 - 1.70)	0.061	114/88	1.23 (0.79 - 1.91)	0.350
	P_{trend}	0.007			0.327			**≤0.005**			0.817			0.062			0.344		
	$P_{interaction}$	**≤0.005**						**≤0.005**						0.169					
Uranium (U)	tert 1 (0.002 — 0.011)	181/362	ref		68/18	ref		37/188	ref		211/192	ref		166/317	ref		81/61	ref	
	tert 2 (0.011 — 0.016)	133/345	0.72 (0.51 - 1.02)	0.071	57/35	0.48 (0.22 - 1.02)	0.057	32/178	0.81 (0.42 - 1.57)	0.544	157/202	0.72 (0.51 - 1.01)	0.063	130/313	0.74 (0.52 - 1.05)	0.099	59/65	0.67 (0.36- 1.24)	0.209
	tert 3 (0.016 — 0.098)	302/224	**3.03 (2.19 -**	**≤0.005**	171/156	**0.32 (0.17 - 0.61**	**≤0.005**	87/173	1.52 (0.84 -	0.163	386/207	**1.71 (1.26 -**	**0.001**	326/262	**2.27 (1.65 -**	**≤0.005**	145/116	0.76 (0.44 -	0.342

			4.20)			**)**			2.76)			**2.34)**			**3.11)**			1.32)	
	P_{trend}	**≤0.005**			**≤0.005**			0.123			**≤0.005**			**≤0.005**			0.400		
	$P_{interaction}$	**≤0.005**						0.052						**≤0.005**					
Manganese (Mn)	tert 1 (0.43 — 0.88)	86/342	ref		29/38	ref		13/194	ref		101/186	ref		81/315	ref		32/64	ref	
	tert 2 (0.88 — 1.41)	126/350	1.49 (0.99 - 2.24)	0.053	58/30	2.49 (1.16 - 5.36)	0.019	33/198	**3.58 (1.49 - 8.56)**	**0.004**	151/182	1.44 (0.98 - 2.14)	0.063	117/311	1.38 (0.92 - 2.08)	0.111	67/67	2.71 (1.34 - 5.49)	0.006
	tert 3 (1.41 — 20.85)	404/239	**7.05 (4.85- 10.24)**	**≤0.005**	209/141	1.91 (1.01 - 3.60)	0.044	110/147	**11.52 (5.10 - 26.00)**	**≤0.005**	502/233	**3.91 (2.76 - 5.54)**	**≤0.005**	424/266	**5.63 (3.93 - 8.06)**	**≤0.005**	186/111	**3.57 (1.87 - 6.82)**	**≤0.005**
	P_{trend}	**≤0.005**			0.157			**≤0.005**			**≤0.005**			**≤0.005**			**≤0.005**		
	$P_{interaction}$	**≤0.005**						**≤0.005**						**≤0.005**					
Copper (Cu)	tert 1 (0.96 —	82/226	ref		58/154	ref		21/157	ref		119/223	ref		84/254	ref		55/124	ref	

	1082.29)																		
	tert 2 (1082.91 — 1259.84)	76/337	0.72 (0.45 - 1.15)	0.177	42/43	**2.68 (1.44 - 5.00)**	**0.002**	24/177	3.32 (1.37 - 8.03)	0.007	94/203	1.11 (0.73 - 1.69)	0.610	80/306	1.31 (0.83 - 2.08)	0.243	37/70	1.37 (0.69 - 2.70)	0.363
	tert 3 (1260.141 — 1975.51)	458/368	**4.64 (3.14 - 6.84)**	**≤0.005**	196/12	**58.49 (27.58 - 124.05)**	**≤0.005**	111/205	**13.32 (6.03- 29.37)**	**≤0.005**	541/175	**9.88 (6.80 - 14.34)**	**≤0.005**	458/332	**8.35 (5.58 - 12.50)**	**≤0.005**	193/48	**18.44 (9.71 - 35.02)**	**≤0.005**
	P_{trend}	**≤0.005**			**≤0.005**			**≤0.005**			**≤0.005**			**≤0.005**			**≤0.005**		
	$P_{interaction}$	**CNA**						**CNA**						**CNA**					
Selenium (Se)	tert 1 (0.484 — 99.37)	344/305	ref		175/75	ref		93/191	ref		425/189	ref		354/295	ref		162/83	ref	
	tert 2 (99.39 — 118.68)	150/309	**0.35 (0.25 - 0.49)**	**≤0.005**	69/71	**0.43 (0.26 - 0.71)**	**0.001**	31/173	**0.29 (0.16 - 0.53)**	**≤0.005**	188/207	**0.40 (0.29 - 0.54)**	**≤0.005**	150/303	**0.32 (0.23 - 0.44)**	**≤0.005**	69/75	0.52 (0.31 - 0.86)	0.012
	tert 3 (118.68 —	122/317	**0.24 (0.1**	**≤0.005**	52/63	**0.40 (0.24 -**	**≤0.005**	32/175	**0.25 (0.1**	**≤0.005**	141/205	**0.29 (0.2**	**≤0.005**	118/294	**0.25 (0.1**	**≤0.005**	54/84	**0.38 (0.2**	**≤0.005**

	213.81)		**7 - 0.34)**			**0.67)**			**3 - 0.48)**			**1 - 0.41)**			**8 - 0.35)**			**2 - 0.65)**	
	P_{trend}	**≤0.005**			**≤0.005**			**≤0.005**			**≤0.005**			**≤0.005**			**≤0.005**		
	$P_{interaction}$	0.096						0.395						0.055					

Abbreviations: GBC, Gallbladder cancer; OR, Odds Ratio; CI, Confidence Interval; tert, tertile; CNA, Conversion not achieved; MDL, Method detection limit, ppb, parts per billion, µl, microliters, µg/L, microgram per liter;

[#]Exposure categories were derived from MDL and 99[th] percentile using control group

[†] - MDL is calculated using standard deviation of all method blanks multiplied by a factor of 3.33. Weighted MDL is derived from individual MDL values to calculate MDL using weighted average method. The weighted average MDLs were 0.961 µg/L for Cr, 0.69 µg/L for Ni, 0.068 µg/L for As, 0.032 µg/L for Cd, 0.263 µg/L for Pd, 0.003 µg/L for U, 0.613 µg/L for Mn, 1.357 µg/L for Cu and 0.684 µg/L for Se).
- Values below the MDL were substituted with MDL/√2 (Cr = 0.680, Ni = 0.488, As = 0.048, Cd = 0.023, Pb = 0.186, U = 0.002, Mn = 0.434, Cu = 0.960, Se = 0.484 µg/L) before log transformation and multivariable logistic regression analyses for OR estimation.
[†1]ORs were adjusted for Age (continuous), Birth residential risk zones (High-risk region/Low-risk region), Any tobacco use (Yes/No), Mustard Oil consumption (Yes/No), Self-reported Gallstone (Yes/No), Education (<5 years of education/≥5 years of education)
[†2]ORs were adjusted for Age (continuous), Sex (Female/Male), Any tobacco use (Yes/No), Mustard Oil consumption (Yes/No), Self-reported Gallstone (Yes/No), Education (<5 years of education/≥5 years of education)
[†3]ORs were adjusted for Age (continuous), Sex (Female/Male), Birth residential risk zones (High-risk region/Low-risk region), Mustard Oil consumption (Yes/No), Self-reported Gallstone (Yes/No), Education (<5 years of education/≥5 years of education)

[§]Bonferroni correction was applied to adjust p-values.

P-trend values calculated from underlying categorical log metal values, by treating them as continuous values in multivariable logistic regression model with confounder adjustment
P for interaction was calculated using continuous log metal values and their interaction with respective confounding variable using Log-likelihood ratio test.

Supplementary Table S21: Odds ratio calculated for log transformed serum metal concentration of Cadmium, Manganese and Uranium.				
Metals*	**Categories (ug/L)/ppb[#]**	**Case/Control**	**OR[†] (95% CI)**	**p-value[§]**
Cadmium (Cd)	tert 1 (0.02 — 0.02)	191/627	**ref**	
	tert 2 (0.03 — 0.04)	88/133	**1.88 (1.26 - 2.80)**	**≤0.005**
	tert 3 (0.04 — 0.24)	633/380	**3.22 (2.39 - 4.33)**	**≤0.005**
Manganese (Mn)	tert 1 (0.43 — 0.88)	115/380	**ref**	
	tert 2 (0.88 — 1.40)	184/380	1.52 (1.06 - 2.17)	0.021
	tert 3 (1.40 — 20.85)	613/380	**3.09 (2.13 - 4.47)**	**≤0.005**
Uranium (U)	tert 1 (0.00 — 0.01)	249/380	**ref**	
	tert 2 (0.01 — 0.01)	190/380	0.65 (0.47 - 0.90)	0.010
	tert 3 (0.01 — 0.09)	473/380	0.70 (0.51 - 0.98)	0.038

Abbreviations: GBC, Gallbladder cancer; OR, Odds Ratio; CI, Confidence Interval; tert, tertile; MDL, Method detection limit; ppb, parts per billion, µl, microliters, µg/L, microgram per liter

*For cases the correlation between Mn and Cd was 0.52, and between Mn and U was 0.80. For controls, these correlations were 0.57 and 0.52, respectively. Therefore, we conducted a sensitivity analysis to adjust for these three metals as categorical variables using multivariate unconditional logistic regression, along with the previously stated confounders.

[#]Exposure categories were derived from MDL and 99th percentile using control group

[†] – MDL is calculated using standard deviation of all method blanks multiplied by a factor of 3.33. Weighted MDL is derived from individual MDL values to calculate MDL using weighted average method. The weighted average MDLs were 0.032 ug/L for Cd, 0.613 ug/L for Mn and 0.003 ug/L for U).

–Values below the weighted MDL were substituted with MDL/√2 (Cd = 0.023, Mn = 0.434, U = 0.002 µg/L) before log transformation and multivariable logistic regression analyses for OR estimation.

– ORs were adjusted for Age (continuous), Sex (Female/Male), Birth residential risk zones (High-risk region/Low-risk region), Any tobacco use (Yes/No), Mustard Oil consumption (Yes/No), Self-reported Gallstone (Yes/No), Education (<5 years of education/ ≥5 years of education)

[§]Bonferroni correction was applied to adjust p-values.

Supplementary Table S22: Association of metal mixture exposure with gallbladder cancer risk using Qgcomp and WQS regression models for Gender						
Models	**Qgcomp**			**WQS**		
Sex	**β (95% CI)**	**OR (95% CI)**	**P value***	**β (95% CI)**	**OR (95% CI)**	**P value***
Male	0.144 (0.026–0.262)	1.16 (1.03–1.30)	**0.016**	1.963 (1.499–2.427)	7.12 (4.48–11.32)	**<0.001**
Female	0.360 (0.255–0.466)	1.43 (1.29–1.59)	**<0.001**	2.080 (1.759–2.401)	8.01 (5.81–11.03)	**<0.001**

Abbreviation: OR: odds ratio; 95% CI: 95% confidence interval; β, Beta; Qgcomp, Quantile g computation; WQS, Weighted quantile sum.

-OR were adjusted for Age (continuous), Birth residential risk zones (High-risk region/Low-risk region), Any tobacco use (Yes/No), Mustard Oil consumption (Yes/No), Self-reported Gallstone (Yes/No), Education (<5 years of education/ ≥5 years of education)

Supplementary Table S23: Review of literature													
Sr No	**PMID**	**Population studied**	**Type of study**	**Year of Publication**	**Sample Type**	**Total No of Cases**	**Total No of Controls**	**Measure of Association**	**Exposure**	**Instrument for Exposure Assessment**	**Confounders**	**Results (Description of Estimates)**	**Outcomes**
1	**9804716**	India (Varanasi)	Case Control Study	1998	Bile (10ml)	96 (GBC)	58 (Gallstones)	Mean ± SE of GBC and control group, Difference of means (95% CI) and p-value	Cadmium, Chromium and Lead	Atomic absorption spectrophotometer (Perkin Elmer-2380)	N. A	**Cadmium**: 0.19 (SE 0.07) mg/l vs 0.09 mg/l, difference 0.10 (0.08 to 0.12), t = 11.63, P < 0.001; **chromium**: 1.26 (0.06) mg/l vs 0.55 mg/l, difference 0.71 (0.58 to 0.84), t = 9.84, P < 0.001; **lead**: 58.38 (1.76) mg/l v 3.99 mg/l, difference 54.4 (50.7 to 58.0), t = 30.07, P < 0.001	Mean biliary concentrations of Cd, Cr and Pb were significantly higher in patients with GBC than with gall stones

2	**23942528**	India (Varanasi)	Prospective Case Control Study	2013	Serum, Bile, gallstones and gallbladder tissues	30 (GBC)	30 (Sex-matched GSD)	Mean concentrations of metals ± SD and the concentration ranges	Chromium, Manganese, Nickel, Copper, Selenium, Lead Cadmium and Zinc	Atomic absorption spectrophotometer (Perkin Elmer-2380)	N. A	**Serum** Concentrations: **Se**: 5.84 **µg/dL** (3.12-9.18), **Zn**: 69.99 (38.04-112.20), **Cd**: 0.65 (0.45-0.86), **Cu**: 159.19 (120.30-206.50), **Mn**: 4.86 (2.68-7.13), **Ni**: 5.08 (2.30-7.50), **Pb**: 4 (1.11-7.51) and **Cr**: 1.01 (0.84-1.93)	**Serum** concentrations of chromium, nickel, copper, and cadmium were also significantly higher in the cancer group | Heavy metals (e.g., chromium, lead, copper, cadmium) were present at significantly higher concentr

													ations in the **gallbladder tissue** from the cancer group, whereas nickel and manganese showed no significant differences | Analysis of **bile** for heavy metals showed significantly higher concentrations of cadmiu

													m, copper, manganese, and nickel in the cancer group.
3	**22502665**	India and Japan	Comparative Study	2012	Fresh tissue samples (GBC and cholecystitis)	India: 8 GBC and 5 cholecystitis | Japan: 10 6 GBC and 4 cholecystitis	N. A	Metal concentration (Min, Max, Median) reported in µg/g, p-value - <0.025	Chromium, Cadmium, Lead, Arsenic, Zinc, Mercury	Transmission Electron microscope (TEM) and Inductively coupled plasma mass spectrometry (Agilent-7500)	N. A	**GBC**: **Cr**-India 1.5, Japan 0.2 µg/g (p-value: 0.002), **Cd**-0.2, 0.1 (0.8), **Pb**- 0.6,0.1 (0.016), As-4.8, 0 (0.002), Zn-110,15 (0.002) and Hg-0.02, 0.01 (0.28) **Cholecystitis**: **Cr**-India 6.1, Japan 0.7 µg/g (p-value: 0.016), **Cd**-0.2, 0.4 (0.19), **Pb**- 0.8,0.1 (0.016), **As**- 1.2, 0.1 (0.016), **Zn**-100,40 (0.11) and **Hg**-0.03, 0.02 (0.29)	Cr, Pb, As and Zn showed higher median levels in Indian GBC as compared to Japan.

4	**40172547**	India (Assam)	Comparative analysis between benign and malignant gallstones	2025	Gallstones	40 Gallstones (10 GBC and 30 GSD) | 10 GBC and 10 GSD gallstones were subjected to elemental analysis	N. A	Mean ± SD, Median for elemental values	Chromium, Arsenic, Selenium, Cadmium, Lead, Vanadium, Strontium, Mercury, Iron, Cobalt	Inductively coupled plasma mass spectrometry (Agilent-7900)	N. A	Median Values of elements in **GBCGS (µg/g)**: **Cr** - 103.28, **As** - 1.18, **Se** - 5, **Cd** - 2.74, **Pb** - 18.92, **V** - 11.37, **Sr** - 43.74, **Hg** - 8.13, **Fe** - 3004.24, **Co** - 3.58 Median Values of elements in **GSD (µg/g)**: **Cr** - 55.44, **As** - 0.99, **Se** - 1.2, **Cd** - 1.3, **Pb** - 6.62, **V** - 6.54, **Sr** - 13.67, **Hg** - 4.42, **Fe** - 1481.57, **Co** - 3.31	GBCGS group revealed significantly higher concentrations of arsenic, chromium, mercury, iron, and lead ($p < 0.05$)

5	**N. A**	India (Delhi)	Observational Case-Control Study	2025	Blood and Urine	70 Cases (35 GBC, 35 GSD)	10	Mean concentrations of metals (µg/L and µg/dL-Pb) and significant difference between groups with p-value <0.05	Magnesium, Titanium, Vanadium, Chromium, Manganese, Iron, Cobalt, Nickel, Copper, Zinc, Arsenic, Selenium, Strontium, Silver, Cadmium, Antimony, Mercury (201),	Inductively coupled plasma mass spectrometry (Agilent-9800)	N. A	Element | GBC | GSD | Control | P-value: Cr 57.42±47.13:16.86±34.28:0.83±2.63:<0.001 | Mn 20.06±10.80:19.03±12.15:6.68±2.19:<0.001| Ni 0.00±0.00:0.0±0.18:0.00±0.00:0.526 | Cu 1211.03±288.59:1137.54±240.41:837.72±102.17:<0.001 | As 0.08±0.36 :0.01±0.05:0.66±0.67:<0.001 | Se 106.33±33.69:129.87±27.46:158.79±21.91:<0.001 | Cd 0.61±1.05:0.85±0.54:0.32±0.38:0.010 | Pb 18.24±20.81:40.93±47.02:39.48±12.06:0.003	Blood and urinary concentrations of **chromium** were significantly higher in GBC patients as compared to GSD patients and control, whereas **manganese and zinc** levels were higher in patients with biliary

									Mercury (202), Thallium, and Lead				pathology (GBC and GSD). **Arsenic and antimony levels were lower** in patients with biliary pathology as compared to control group.

6	**27888389**	India (West Bengal)	1. Case Control study 2. Case Series	2016	Gallstones	46	65	Mean ± SD, Elemental values in µg/g, p-value <0.05	Mercury, Lead, Arsenic, Cadmium, Chromium and Cobalt	Atomic absoprtion spectrophotometer	N. A		Mercury and Lead were higher in premaligant and malignant group whereas Arsenic, chromium, cobalt, cadmium were presented in all types of gallstones in both groups Cadmium was found higher in cholesterol

													stones in PM-M group as compare to C-C group.

7	**36918592**	India (Bihar)	1. Case Control study 2. Case Series 3. Ecological Study	2024	GB Tissue, Gallstone, Bile, Blood, Hair were collected for Arsenic estimation.	**N = 152** (GB Tissue,GB Gallstone, Bile, Blood,Hair) | **N = 512** (Blood)	N = 200	Mean +- SE., Maximum Value., Minimum Value., 95% Confidence Interval of Mean	Arsenic	Atomic Absorption Spectrophotometer	N. A	Biological samples studied| Arsenic concentration (mean ± S.E) | Maximum arsenic concentration | Minimum arsenic concentration | Lower 95% CI of mean | P value 1.GBC Patient's Tissue Arsenic Concentration (µg/Kg) (n = 152) 340.6 ± 31.98 | 2166 | 0.6700 | 277.4 | < 0.0001 2. GBC Patient's Gallstone Arsenic Concentration (µg/Kg) (n = 152) 78.41 ± 8.311 | 634.9 | 0.0300 | 61.99 | < 0.0001 3. GBC Patient's Bile Arsenic Concentration (µg/L) (n = 152) 59.10 ± 6.554 | 483.2 | 0.0230 | 46.15 | < 0.0001 4. GBC Patient's Blood Arsenic Concentration (µg/L) (n = 152) 52.28 ± 5.504 | 389.3 | 0.2000 | 41.41 | < 0.0001 5. GBC Patient's Hair Arsenic Concentration (µg/Kg) (n = 152) 649.7 ± 93 |88 6980.1 | 0.2000 | 464.2 | < 0.001	HPR Confirmed GBC

												6. Control Subject's Blood Arsenic concentration (µg/L) (n = 200) 0.8133 ± 0.1670 | 11.70 | 0 0.4840 | < 0.001 Control Subject's Hair Arsenic Concentration (µg/Kg) (n = 200) 1.541 ± 0.2351| 14.40 | 0 1.077 | < 0.0001	
8	**31145133**	India, USA, Taiwan (selected on availability of	Observational Ecological Study	2020	NA	NA	NA	As in ground water and it's role in GBC incidence	Groundwater Arsenic	NA	Stratified by Sex	Among women, correlations were observed worldwide (Spearman = 0.31, P = 0.028), in Taiwan (R2 = 0.57, P = 0.005) and in India (R^2 = 0.23, P = 0.006). In men, a correlation was observed in India (R^2 = 0.26, P = 0.009) and a modest correlation was identified in	This study provides supports to the hypothesis that high-level

		Data)										the USA (R^2 = 0.14, P = 0.026).	exposure to As in drinking water may be associated with GBC risk
9	**36622765**	India (Assam and Bihar)	Case Control Study	2023 - 03	Groundwater monitoring data collected from 192694 wells across 71 districts of Assama and Bihar (Central Groundwater	214 (Biopsy confirmed)	166 (Unrelated)	Continuous - means [t-test] or medians [wilcoxin rank sum], categorical - percentage, bivariate comparisons for characterstict of	Arsenic	Ultraviolet spectrophotometry, atomic absorption spectrophotometry	age, sex, state, education, montly household income, betel-nut, alcohol, fruit /vegetables consumption, energy intake estimation,	Ground Water Weighted concentration: Tertile 2 : (2.00, 95% CI: 1.05–3.79) Tertile 3 :(2.43, 95% CI, 1.30–4.54) in comparison tertile 1 |	Our findings provide preliminary but suggestive evidence for the association of arsenic in drinking water and gallbladder cancer risk

					Board for the state of Assam and Bihar)			individuals - chi-sq test, multivariable analysis.			physical activity, exposure to passive smoking and waist circumference		
10	**36509118**	Chile	Retrospective Case Control Study	2023	GB Tissue stored in Formalin fixed paraffin embedded (FFPE) blocks	35	42	Measuring and comparing As concentration in tissue samples from patients with GBC vs NCGB enrolled at hospital	Arsenic	Inductively coupled Plasma - Mass Spectrometer connected to HPLC (Speciation of Arsenic was done on 24 GBC and 4 NCGB patients)	Gender, Age, Gallstones	The comparison between gallbladder cancer (GBC) and non-cancerous gallbladder (NCGB) patients shows: Gender: Similar proportion of females in both groups (74.3% for GBC vs. 76.2% for NCGB, p = 1.000). Age: GBC patients are older (median age 67.5 years vs. 49 years for NCGB, p < 0.001). Gallstones: Slightly lower prevalence in GBC patients (86.2% vs. 95.2% for NCGB, p = 0.218).	High As levels in Cases as compared to controls

11	**31318976**	China (Shanghai)	Population Based Case Control Study	2020	Serum Fractions (atleast 120ul)	259 GBC patients, 701 Patients with Gallstones (960)	851	Logistic Regression models used to estimate adjusted OR with 95% CI for association. t-tests for difference in means for continuous variables and chi-squared tests for categorical variabl	18 detectable analytes, including arsenic, boron, cadmium, calcium, chromium, cobalt, copper, iron, lithium, magnesium, manganese, molybdenum, nickel, phosphorous,	Inductively coupled Plasma - Mass Spectrometry	Adjustment for age, sex, body mass index (BMI), cigarette smoking, alcohol consumption, and levels of triglycerides and cholesterol	Cadmium, chromium, copper, and molybdenum levels were higher in GBC patients compared to gallstone patients. Arsenic, boron, lithium, sulfur, iron, magnesium, and selenium levels were lower in GBC patients	This study demonstrates the value of metallomics as a novel approach to further explore the role of metals, which have an extensive and essential role in the pathophysiology and pathogenesis of gallbladder diseases.

								es. Serum levels of metals were categorized by tertiles (Ts) and compared for differences by the two groups (GBC vs. gallstones and gallstones vs. controls) by chi-squared tests with Bonfer	seleniu m, sulfur, vanadi um, and zinc.				

								roni correct ions.					

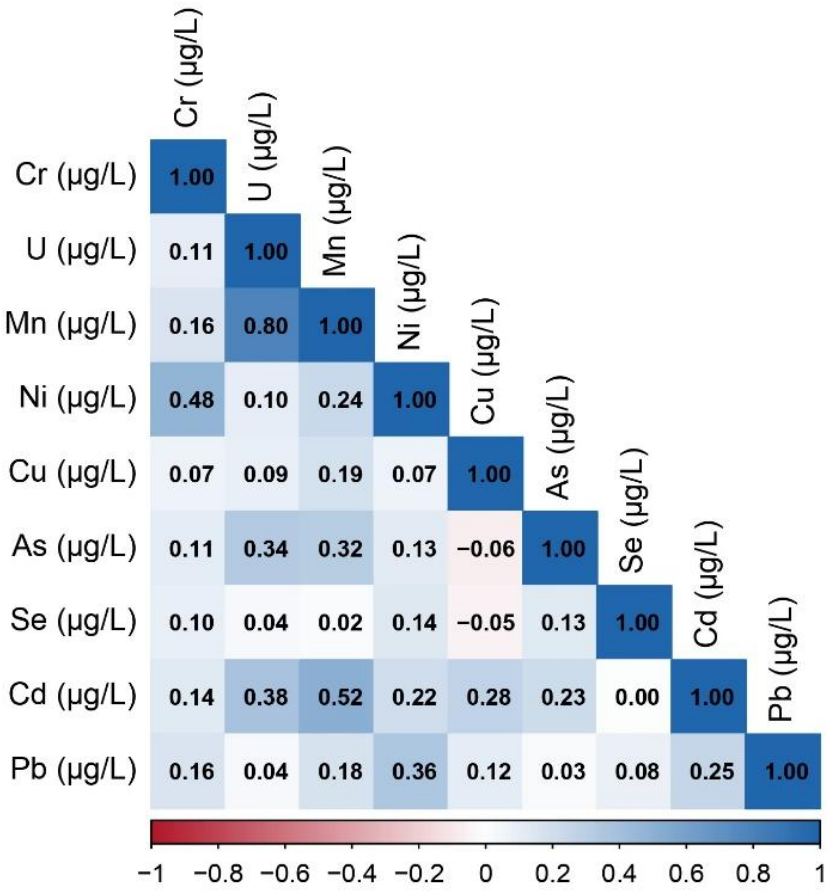
Supplementary Table S24: Sources of Metal exposure						
Sr No.	**Element**	**Natural Sources**	**Dietary Sources**	**Anthropogenic Sources**	**Exposure Pathways**	**References**
1	Chromium (Cr)	Geological Leaching of Cr enriched rocks	Consumption of contaminated crops and water	Metal Processing & Plating, Stainless steel manufacturing, welding fumes, Leather Tanning, Textile & Dyeing, Chemical Manufacturing, Cement & Refractory Industries, Combustion, cigarette smoke	Inhalation of dust, Drinking contaminated water	1. PMID: 41373804; 2. PMID: 33927623
2	Manganese (Mn)	Present in rocks, minerals, and soils Naturally enters groundwater and surface water Released due to changes in pH and oxygen (redox conditions) Mobilized by microbial activity	Contaminated whole grains, nuts, tea, leafy vegetables	Mining and ore processing; Industrial dust; Steel, iron, and ferroalloy industries; Battery manufacturing; Burning of fossil fuels and waste (airborne Mn particles); Municipal wastewater and sewage sludge Minor contributions from fertilizers and pesticides	Food, inhalation	1. PMID: 41373804; 2. DOI: 10.5772/intechopen.1013063
3	Nickel (Ni)	Earth's crust; soil	Contaminated	Stainless steel, batteries,	Occupational air	1. PMID: 41373804;

			nuts, chocolate, grains, legumes, soya products (tofu, soymilk), baking powder, cocoa	electroplating, welding, fossil fuel combustion, orthodontic braces, jewellery and metal alloys causing dermal exposure	exposure; food	2. DOI: 10.5772/intechopen.1013063.
4	Copper (Cu)	Weathering of copper-containing rocks and soil Natural dust containing copper Release from oceans Volcanic and atmospheric particulates Decaying vegetation	Shellfish, seafood, liver, nuts, whole grains	Mining, smelting, corrosion of copper pipes, Mining and processing of copper ores (dust & wastewater) Electronics and alloy manufacturing effluents Copper-based fungicides in agriculture (vineyards, orchards) Sewage treatment plants and domestic wastewater Burning of fossil fuels and waste Wear of brake pads and copper wiring Leaching from copper plumbing, especially in acidic or hot water Landfills and improper disposal of metal scrap	Drinking contaminated water (especially from corroded copper pipes) Consumption of food grown in contaminated soil (e.g., liver, crops) Inhalation of copper-containing dust and industrial emissions Ingestion of contaminated soil, particularly in children	1. PMID: 41373804; 2. DOI: 10.5772/intechopen.1013063.
5	Arsenic (As)	Geological deposits; arsenic-rich groundwater; volcanic regions	Contaminated rice and rice products; seafood (organic As); contaminated drinking water	Mining; smelting; pesticides; wood preservatives; coal combustion, metal adhesives; industrial waste	Drinking water; food	1. PMID: 41373804; 2. PMID: 33927623
6	Selenium (Se)	Se-rich soils;	Brazil nuts,	Coal combustion; mining; metal	Inhalation, consumption	1. PMID: 41373804;

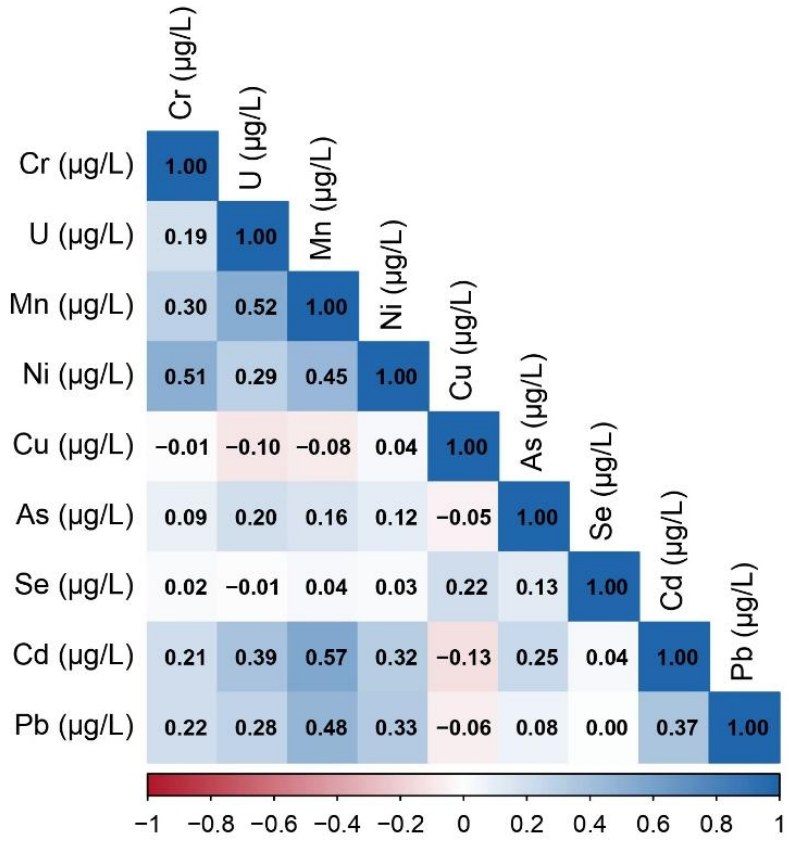
		volcanic activity	seafood, meat, cereals	refining industries; agriculture runoff; electronics and glass manufacturing	of contaminated water or crops	2. DOI: 10.5772/intechopen.1013063.
7	Cadmium (Cd)	Groundwater contamination by industrial wastewater effluents	1. Certain plants (tobacco, contaminated rice and other cereal grains, potatoes) 2. Meat, Shellfish, Mushrooms	Metal plating, Pigment production, Ni-Cd batteries, Stabilizers in plastics, Neutron absorbent in nuclear reactors, Use of Phosphate fertilizers, Vehicle emissions, mining and smelting	Diet, Smoking, Inhalation and Ingestion	1. PMID: 22945569. 2. DOI: 10.3390/w14213432
8	Lead (Pb)	Earth's crust; soil	Spices like turmeric, chilly powder, oysters	Lead-acid battery industries, Lead-based paints, stained glass making, plumbing system,vehical-related pollution, ceramics, jewellery, toys	Ingestion, Inhalation, consumption	1. PMID: 33927623 2. DOI: 10.3390/w14213432
9	Uranium (U)	Geographical (Lignite, granite, phosphate rocks), groundwater leaching and volcanic eruptions	Drinking water and root vegetables	U mining and milling, nuclear power plants, coal burning, phosphate fertilizers	Ingestion of drinking water	1. PMID: 41373804

Supplementary Table S25: Optimal biological specimen for metal analysis					
Metals	**Suitable Matrix**	**Biospecimens Used**	**Rationale / Comments**	**PMID**	**Reference**
Chromium (Cr)	Blood, Urine	Serum	Best biomarker for recent Cr exposure, especially Cr (VI)	**21211822**	Gil et al., 2011 (PubMed)
Manganese (Mn)	Whole Blood	Serum	Preferred biomarker for environmental and occupational exposure	**37337116**	Martinez-Morata et al., 2023 (PubMed)
Nickel (Ni)	Urine	Serum	Urinary Ni reflects recent exposure better than blood	**37337116**	Martinez-Morata et al., 2023 (PubMed)
Copper (Cu)	Serum	Serum	Standard clinical biomarker; reflects Cu status	**37337116**	Martinez-Morata et al., 2023 (PubMed)
Arsenic (As)	Urine	Serum	Gold-standard biomarker for recent arsenic exposure	**37337116**	Martinez-Morata et al., 2023 (PubMed)
Selenium (Se)	Serum / Whole Blood	Serum	Reflects nutritional status and long-term exposure	**37337116**	Martinez-Morata et al., 2023 (PubMed)
Cadmium (Cd)	Urine	Serum	Urine reflects cumulative body burden; blood reflects recent exposure	**37337116**, **21211822**	Martinez-Morata et al., 2023; Gil et al., 2011 (PubMed)
Lead (Pb)	Whole Blood	Serum	Internationally accepted biomarker of lead exposure	**37337116**, **21211822**	Martinez-Morata et al., 2023; Gil et al., 2011 (PubMed)
Uranium (U)	Urine	Serum	Preferred biomarker due to rapid renal excretion	**37337116**	Martinez-Morata et al., 2023 (PubMed)

Supplementary Figure S1: Spearman's Correlation Matrix of Metal Concentrations (µg/L) in GBC Cases and Controls



Correlation matrix: Cases



Correlation matrix: Controls

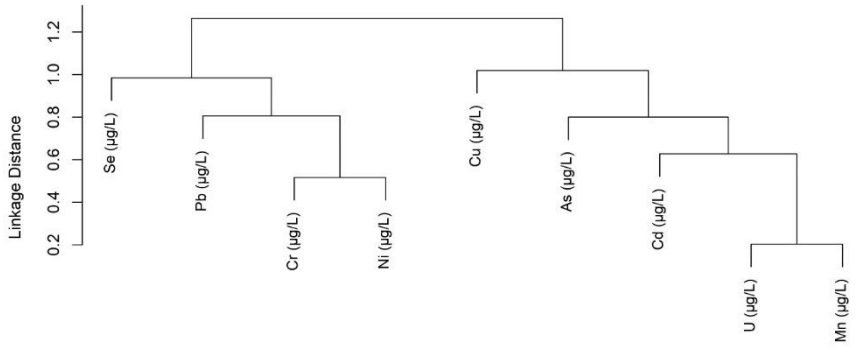
Supplementary Figure S1: Spearman's rank correlation matrix showing pairwise associations between metal concentrations (µg/L) among gallbladder cancer (GBC) cases and controls. Original (untransformed) metal concentrations were used to evaluate inter-metal relationships.

In GBC cases, a very strong positive correlation was observed between uranium (U) and manganese (Mn) (r = 0.80). Moderate correlations were noted between cadmium (Cd) and manganese (Mn) (r = 0.52), and chromium (Cr) and nickel (Ni) (r = 0.48).

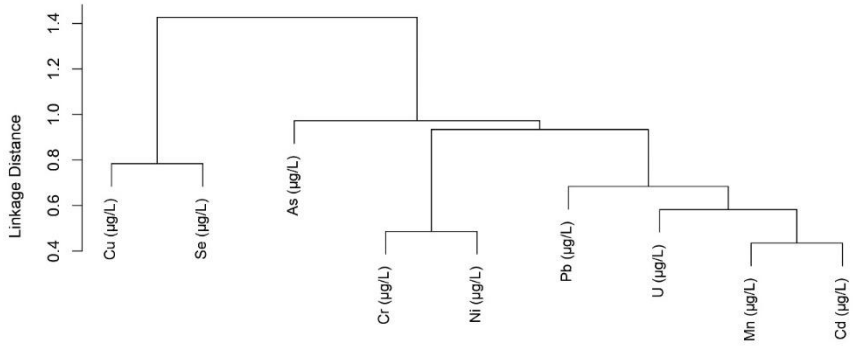
In the control group, moderate positive correlations were observed between chromium (Cr) and nickel (Ni) (r = 0.51), uranium (U) and manganese (Mn) (r = 0.52), cadmium (Cd) and manganese (Mn) (r = 0.57), lead (Pb) and manganese (Mn) (r = 0.48), and nickel (Ni) and manganese (Mn) (r = 0.45).

Overall, most metals exhibited weak correlations with cadmium (Cd) across both case and control groups.

Supplementary Figure S2: Hierarchical Clustering of Heavy Metals Based on Correlation-Derived Distance in GBC Cases and Controls



Hierarchical clustering analysis for metals in GBC cases



Hierarchical clustering analysis for metals in controls

Supplementary Figure S2: Dendrogram representing hierarchical clustering of serum metals (µg/L) among gallbladder cancer (GBC) cases (n=912) and controls (n=1140) using a correlation-derived distance matrix (1 − correlation) and Ward's linkage method.