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The biliary and urinary excretion pattern of toxic and essential metals in patients with biliary and liver diseases and its association with fibrosis-4 index

Hamad Alashgar[1], Ali Albenmousa[2], Musthafa Peedikayil[1], Mahmoud Abougamel[1], Abdulrahman Alfadda[1], Fahad Alsohaibani[1], Khalid Alkahtani[1], Bader AlAjlan[1], Faisal Abaalkhail[1], Iman Al-Saleh[3]*

[1] Gastroenterology Section, Department of Medicine, [2] Transplant Endoscopy Unit, Liver and Small Bowel Health Centre, [3] Environmental Health Program, [1,2,3] King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia

Abstract

This study aimed to explore the urinary and biliary excretion of toxic and essential metals in 60 patients diagnosed with liver and biliary diseases and their potential association with hepatic fibrosis. The levels of mercury (Hg), lead (Pb), cadmium (Cd), arsenic (As), selenium (Se), manganese (Mn), copper (Cu), and zinc (Zn) in patients' urine and bile samples were determined using inductively coupled plasma-mass spectrometry. To assess hepatic fibrosis, we calculated the Fibrosis-4 (FIB-4) index, a non-invasive measure, using a formula incorporating age, aspartate aminotransferase, alanine aminotransferase, and platelet levels. The median values of biliary (urinary) Hg, Pb, Cd, As, Se, Mn, Cu, and Zn in $\mu g.L^{-1}$ were 2.7 (3.6), 1.6 (0.2), 0.126 (1.3), 0.319 (6.3), 14.1 (43.7), 43.4 (4.5), 244.3 (82.9), and 201.6 (692.5), respectively. Biliary levels of Pb, Mn, and Cu were significantly higher than those in urine (p for all <0.001), suggesting that these metals are primarily eliminated through biliary excretion. There was a clear imbalance in the biliary Cu and Zn levels; 33 (~57%) patients had a Cu/Zn ratio >1. FIB-4 index >1.3 was found in 28 patients at risk of liver fibrosis. Analysis of covariance revealed patients with FIB-4 index >1.3 had higher urinary levels of Pb (more than threefold) and Zn (approximately twofold) higher than those with lower FIB-4 scores. Conversely, patients with higher FIB-4 scores had significantly lower biliary levels of Mn (approximately fourfold) and Cu (more than twofold). These findings suggest that imbalances in certain metals may play a pivotal role in the pathophysiology of hepatic fibrosis, warranting further exploration.

Keywords:
toxic metals, essential metals, bile, urine, liver fibrosis

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*Correspondence:
Dr. Iman Al-Saleh (MBC#03)
Environmental Health Program
King Faisal Specialist Hospital & Research Centre
PO Box: 3354, Riyadh 11211, Saudi Arabia
E-mail: iman@kfshrc.edu.sa

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1 Introduction

The liver produces bile; it consists mainly of salts, phospholipids, cholesterol, conjugated bilirubin, electrolytes, and water and is stored in the gallbladder [1]. Biliary excretion is crucial for eliminating drugs and their metabolites, nutrients, and xenobiotics [2, 3]. Bile formation has a vital role in health,

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and impairment of its secretion can cause cholestasis, characterized by obstruction of bile flow followed by hepatocyte injury [4]. Cholestatic liver injury often progresses to liver fibrosis, cirrhosis, and liver failure [5].

The limited available studies have reported that many essential metals (iron (Fe), manganese (Mn), magnesium, chromium (Cr), zinc (Zn), copper (Cu), nickel, and cobalt) are excreted into hepatic bile [6]. Studies have found associations between trace metal status and severity of liver cirrhosis [7, 8] and fibrosis [9, 10]. The involvement of deficiency or excess of trace metals in the pathogenesis of chronic liver diseases has been documented [11].

Heavy metals such as cadmium (Cd), mercury (Hg), lead (Pb), and arsenic (As) are widespread contaminants that have generated considerable attention because they are nondegradable and come from natural and anthropogenic sources [12]. The general population may be exposed to heavy metals from various sources such as air, food, drinking water, smoking, cosmetics, dental amalgam, and other domestic sources [13, 14]. Heavy metals accumulate in body organs over a long exposure period, posing serious health problems [15]. The liver has a major role in the metabolism of xenobiotic compounds and, therefore, becomes susceptible to chemical injury [16]. Accumulation of heavy metals in the liver has been documented as disturbing various hepatic pathways via oxidative stress, lipid peroxidation, changes in cholesterol metabolism, hyperplasia, and cell death [17, 18].

Essential metals such as Zn, Cu, selenium (Se), chromium, Fe, and Mn are crucial in various physiological and biochemical activities [19]. The liver is essential to maintaining the homeostasis of trace metals [20]. Therefore, their metabolism can be altered in hepatic disorders [11, 21] toward excess or deficiency of the metal [22]. The role of metabolic abnormalities of some trace metals is still unclear, and further understanding of it may have therapeutic implications in delaying or preventing complications of liver diseases [11].

Several non-invasive clinical laboratory-based markers have been developed to detect and assess the severity of liver fibrosis [23]. The fibrosis-4 (FIB-4) index has demonstrated its validity in detecting significant liver fibrosis in various chronic liver diseases [24, 25]. The Fibrosis-4 (FIB-4) index has proven its effectiveness in identifying significant liver fibrosis across a range of chronic liver diseases [24, 25]. Several studies have observed a noteworthy inverse correlation between serum zinc (Zn) or copper (Cu) levels and liver fibrosis, as indicated by the FIB-4 index, among patients with non-alcoholic fatty liver disease (NAFLD) [10, 26, 27].

In Saudi Arabia, Alswat [28] documented a substantial prevalence of NAFLD, expected to escalate from 25.1% in 2017 (across all age groups) to 31.7% by 2030. This increase is attributed to elevated rates of obesity and type 2 diabetes, identified as the primary risk factors for the disease. Al-Hamoudi [29] estimated that 10% to 20% of patients with NAFLD would develop advanced liver fibrosis and cirrhosis. Additionally, previous studies have shown that the Saudi population is exposed to metals from various typical and/or unusual sources [30-35].

This study aimed to (a) assess the levels of toxic and essential metals in bile and urine samples taken from patients with biliary and liver diseases and (b) examine their association with liver fibrosis assessed using the FIB-4 index.

2 Materials and methods

2.1 Study participants

Between January 14 and June 1, 2021, we recruited 60 patients (≥18 years old) who were either inpatients or outpatients at King Faisal Specialist Hospital & Research Centre (KFSH&RC) and who needed endoscopic retrograde cholangiography (ERCP) or percutaneous transhepatic cholangiodrainage (PTC) or cholecystectomy. Patients with malabsorption, patients younger than 18, pregnant women, lactating mothers, and patients with diminished mental capacity or altered sensorium were excluded. All patients signed a consent approved by the KFSH&RC Research Ethics Committee (RAC #2201194). Each patient completed a questionnaire that collected data on sociodemographic characteristics, lifestyle, occupation, diet, smoking, and personal medical history.

2.2 Sample collection

Hepatic bile (1.5–7 mL) was collected in acid-prewashed polyethylene tubes during ERCP, PTC, or cholecystectomy procedures. All possible precautions were taken to prevent contamination during the sample collection, and the samples were subsequently stored at −20°C until analysis.

Approximately 25 mL of spot urine sample was collected in a plastic container from each patient on the same day that bile secretions were collected and stored at −20°C until analysis.

A blood sample (5 mL) was drawn from each patient within 48 hours of the ERCP/PTC/cholecystectomy for testing blood

cell count and hepatic profile at the Clinical Biochemistry/Hematology, Pathology and Laboratory Medicine Department of KFSH&RC.

Bile and urine samples were available for 59 and 52 of the 60 patients, respectively.

2.3 Assessment of metals

The bile and urine samples were diluted 10× and 50×, respectively, with an acidic diluent that was a mixture of 0.5% nitric acid (Fisher Scientific, PA, USA), 0.05% Triton-X (Sigma-Aldrich™, MO, USA), and 2% methanol (Fisher Scientific, PA, USA; all v/v). Internal standards added to the urine samples were iridium (^{193}Ir) for Pb, Hg, and Cd; indium (^{115}In) for As; rhodium (^{103}Rh) for Se, Zn, and Cu; and scandium (^{45}Sc) for Mn. For bile analysis, ^{193}Ir was used for Pb, Cd, Hg, and Mn, and ^{103}Rh for Se and As. The quantification of metals in bile and urine samples was performed by inductively coupled plasma-mass spectrometry (ICP-MS; Perkin Elmer NexION®, 2000). The diluent intensity was subtracted from calibrator standards, quality control, and patient samples. The calibration curve ranged between 0.5 and 10 $\mu g.L^{-1}$ for all tested metals except Cu (2–32 $\mu g.L^{-1}$) and Zn (75–1200 $\mu g.L^{-1}$). These ranges were satisfactory, with linear correlation coefficients (r^2) above 0.999. Pooled urine samples were spiked with three different metal levels and ran parallel with patient samples to check between-run precision. The spike recovery values of the metal concentrations were 94%–108%. The sample replicates' relative standard deviation (RSD) was <10% for within- and between-run precision. Two Bio-Rad Lyphochek Urine Metals Control Level 1 (Lot 69161) and Level 2 (Lot 69162) certified reference urine materials (Munich, Germany) and National Institute of Standards and Technology Standard Reference Materials 1643F for trace metals in water (Gaithersburg, MD, USA) were employed to validate the method. Our values fell within the certified range values. The method detection limits (MDLs) for metals in $\mu g.L^{-1}$ were 0.021 (Pb), 0.0035 (Cd), 0.021 (Hg), 0.017 (As), 0.105 (Mn), 0.143 (Se), 0.195 (Cu), and 1.487 (Zn).

2.4 Statistical analysis

Key data characteristics were presented as mean ± SD and minimum-maximum or percentage (%) where appropriate. Geometric mean (GM) and median were used to describe the distributions of urinary and biliary metals. The metals were transformed to the natural logarithm (ln) to approximate a normal distribution. Pearson's correlation analysis (r) was applied to assess associations between pairs of continuous variables. For the binary outcome (FIB4-index), we used standard χ^2-tests and Student's t-tests to test the significance of dichotomous and continuous variables, respectively. We replaced the χ^2-test with a two-tailed Fisher's exact test in 2×2 tables if the expected frequency was <5 in any of the cells.

Additionally, an analysis of covariance (ANCOVA) was performed to examine differences in the levels of each metal between the two groups of patients (FIB4-index ≤1.3 and >1.3). Post hoc comparisons of means were carried out using the least significant difference (LSD) test. Risk factors/confounders that were associated with FIB4-index and/or metals ($p < 0.1$) were included in the analysis. Some confounders were excluded due to a limited number of cases in one category versus the others. The antilog of the adjusted mean ± standard error (SE) and 95% confidence interval (95% CI) were reported for each group.

All values below MDL were replaced with ½ MDL. We conducted the statistical analyses using SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, USA). The significance level was set at $p < 0.05$. We also defined $p < 0.10$ as a marginally significant effect due to our study's small sample size and exploratory nature.

3 Results

3.1 Baseline characteristics

Table 1 presents the basic demographic and health data of patients who underwent ERCP for various health reasons. In this study, we enrolled 60 patients (age range 22–81 years, with a median age of 56.5 years). Of the patients, 38 were male, and 22 were female; they were all Saudis, and half had bachelor's degrees. Ten of the 60 patients were smokers, all males. The regional distribution of patients was based on the province from which they originated. Most of our patients came from the central province (37), while the rest came from other provinces: 2 from the eastern province, 7 from the northern province, 4 from the southern province, and 10 from the western province. The median body mass index (BMI) was 26.4 kg/m^2 (17.2–50.4 kg/m^2). Although the median female BMI (27 kg/m^2) was slightly higher than the male value (26 kg/m^2), the difference was not significant (p = 0.19). The prevalence of obesity (BMI ≥30 kg/m^2), overweight (BMI ≥25 kg/m^2), and underweight (BMI <18.5 kg/m^2) among

Table 1. | General characteristics of patients.

Continuous variables	*N*	Mean	SD	Median	Minimum	Maximum	KFSH&RC reference range
Age (years)	60	51.8	15.9	56.5	22.0	81.0	
Weight (kilograms)	60	74.6	19.0	73.5	40.0	129.0	
Height (cm)	60	164.7	9.7	165.0	138.0	183.0	
BMI (Kg/m^2)	60	27.4	6.4	26.4	17.2	50.4	
Hemoglobin (HGB) (g/L)	58	119.5	22.5	121.5	65.0	166.0	135–180
White blood count (WBC) ($\times 10^9$/L)	58	6.6	2.9	6.3	1.9	15.4	3.9–11
Platelets ($\times 10^9$/L)	58	247.9	92.7	248.0	49.0	448.0	155–435
Albumin (g/L)	58	36.6	5.9	37.2	19.4	45.4	28–46
Total bilirubin ($\mu mol.L^{-1}$)	58	59.8	87.4	15.5	2.6	410.1	0–21
Total protein (g/L)	58	65.0	9.0	65.4	40.0	82.5	65–81
Alanine transaminase (ALT) (U/L)	58	82.8	104.7	52.2	5.0	671.7	10–45
Aspartate transaminase (AST) (U/L)	58	57.8	49.2	41.5	6.2	280.6	10–45
Alkaline phosphatase (ALP) (U/L)	58	273.3	191.2	224.0	54.0	908.8	50–116
Fibrosis-4 (FIB-4) index	59	2.0	2.2	1.2	0.3	12.8	
Categorical variables						Count/(percentage %)	
Region of living	60	Central /Other regions				37 (61.7) / 23 (38.3)	
Educational level	60	≤ High school / > High school				30 (50) / 30 (50)	
Gender	60	Male / Female				38 (63.3) / 22 (36.7)	
Smoking	60	Yes / No				10 (16.7) / 50 (83.3)	
Choledocholithiasis	60	Yes / No				14 (23.3) / 46 (76.7)	
Biliary stricture	60	Yes / No				3 (5) / 57 (95)	
Cholangiocarcinoma	60	Yes / No				2 (3.3) / 58 (96.7)	
Pancreatic cancer	60	Yes / No				4 (6.7) / 56 (93.3)	
Primary sclerosing cholangitis	60	Yes / No				6 (10) / 54 (90)	
Post-liver transplant-related biliary complications	60	Yes / No				20 (33.3) / 40 (66.7)	
Gall bladder cancer	60	Yes / No				1 (1.7) / 59 (98.3)	
Cirrhosis	60	Yes / No				13 (21.7) / 47 (78.3)	
Chronic hepatitis B infection	60	Yes / No				3 (5) / 57 (95)	
Wilson's disease	60	Yes / No				1 (1.7) / 59 (98.3)	
Post liver transplant	60	Yes / No				2 (3.3) / 58 (96.7)	
Diabetes Mellitus	60	Yes / No				22 (36.7) / 38 (63.3)	
Hypertension	60	Yes / No				23 (38.3) / 37 (61.7)	
Coronary heart diseases	60	Yes / No				5 (8.3) / (91.7)	
Lung diseases	60	Yes / No				1 (1.7) / 59 (98.3)	
Renal diseases	60	Yes / No				6 (10) / 54 (90)	
Hematological diseases	60	Yes / No				2 (3.3) / 58 (96.7)	
Pancreatic diseases	60	Yes / No				2 (3.3) / 58 (96.7)	
Malignancies	60	Yes / No				5 (8.3) / 55 (91.7)	
Lymphoma	60	Yes / No				1 (1.7) / 59 (98.3)	

our patients was 31.7%, 66.7%, and 5%, respectively [36]. More than 38% and 37% of our patients were hypertensive and diabetic, respectively. Thirteen patients had cirrhosis. The patients were on various kinds of medication at the time of bile collection. More than 50% of the patients were on proton pump inhibitors. The other drugs were ursodeoxycholic acid, mycophenolate mofetil, prednisolone, and tacrolimus. Many patients were also on insulin, oral antidiabetic, and antihypertensive medications.

There were 40, 6, 11, and 27 patients with hemoglobin (HGB), white blood count (WBC), platelets, and total protein lower than the lower KFSH&RC reference limits of 135 g/L, 3.9 × 10^9/L, 155 × 10^9/L, and 65 g/L, respectively. In contrast, 58, 25, 1, 30, 28, and 41 patients had albumin, total bilirubin, total protein, alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) lower than the upper KFSH&RC reference limits of 46 g/L, 21 μmol.L^{-1}, 81 g/L, 45 U/L, 45 U/L, and 116 U/L, respectively.

We calculated the FIB-4 using the following formula: age ([yr] × AST [U/L]) / ((PLT [10^9/L]) × (ALT [U/L])$^{(1/2)}$) developed by Sterling [24]. The authors considered that significant hepatic fibrosis was present when the FIB-4 index was >1.3. In our study, 28 patients had FIB-4 >1.3.

3.2 Distribution of urinary and biliary metals

Figure 1 shows the distribution of urinary and biliary levels of metals. All the metals were detectable in bile samples apart from Hg (~90% >0.021 μg.L^{-1}) and in urine apart from Cd and Pb (~73% and 71% >0.0035 and 0.021 μg.L^{-1}, respectively). Paired *t*-tests indicated significantly higher biliary levels of Pb, Mn, and Cu (p for all <0.001) than in urine. In contrast, Cd, As, Se, and Zn urinary levels were significantly higher than in bile (p for all <0.001), except Cd (p = 0.028). No significant difference was noted in the Hg levels of bile and urine (p = 0.382). Significant positive inter-correlations among certain metals, whether in urine or bile, were observed but with a different pattern (**Table S1**). Apart from the inverse relationship between urinary and biliary Mn levels, no correlations were seen between urinary metals and their counterparts in bile. We observed inter-metal significant negative correlations, particularly between biliary As and urinary Hg, Cd, Mn, Cu, and Zn and between biliary Pb and urinary Se, Mn, Cu, and Zn. Results are shown in **Table S2**.

For confounders, only age was positively and negatively correlated with urinary levels of Pb (p = 0.035) but negatively with arsenic (p = 0.028), Cu (p = 0.043), and the ratio of Cu/Zn (p = 0.031). Males had significantly higher levels of Zn in urine (p = 0.006) and bile than females (r = 0.065). BMI was inversely correlated with urinary Hg (p = 0.009) and Cd (p = 0.034). Biliary Pb levels were significantly higher in patients from the central province (p = 0.006). Patients with high school educational levels or higher had significantly lower levels of urinary Cd (p = 0.021), Cu (p = 0.018), and Cu/Zn ratio (p = 0.023). Biliary Mn was significantly higher in patients with an educational level higher than high school (p = 0.084). Patients who had post-liver transplants related to biliary complications had significantly lower urinary Se levels (p = 0.004) and As (p = 0.059). Patients with cirrhosis had significantly lower biliary As (p = 0.005). Patients with diabetes mellitus had lower urinary As (p = 0.026), Cu (p = 0.06), and Cu/Zn ratio (p = 0.007) and higher urinary Zn levels (p = 0.063). Hypertensive patients had a significantly lower Cu/Zn ratio (p = 0.018). Results are presented in **Table S3**.

3.3 Associations between urinary/biliary metals and FIB4-index

We compared the demographic data, clinical parameters, health risk factors, and levels of metals in urine and bile according to FIB-4 indexes (≤1.3 and >1.3). As shown in **Table 2**, patients with FIB-4 index >1.3 were older (p < 0.001), male (p = 0.05), had higher BMI (p = 0.064), high school educational level or lower (p = 0.036), and were more likely to have cirrhosis (p = 0.038) and diabetes (p = 0.067) but had lower levels of urinary As (p = 0.033), biliary Mn (p = 0.008), and biliary Cu (p = 0.003). In contrast, higher levels of urinary Pb (p = 0.011) and Zn (p = 0.003) were found in patients with a high FIB-4 index (>1.3). We applied the ANCOVA models to test the differences in the levels of each metal between the two groups of patients with low and high FIB4-index. All models significantly associated with FIB4-index in the bivariate analyses at $p \leq 0.1$ were adjusted for age, gender, BMI, educational level, cirrhosis, and diabetes (**Table 2**). In addition, each model was adjusted further for confounders/risk factors related significantly to the metal levels (**Table S3**).

Both urinary Pb and Zn levels remained higher in patients with FIB4-index >1.3, but this was marginally significant. Biliary levels of Mn and Cu remained significantly low in patients with FIB-4 index >1.3. The antilog of the adjusted means ± SE of means for each of the two groups is presented in **Table 3**.

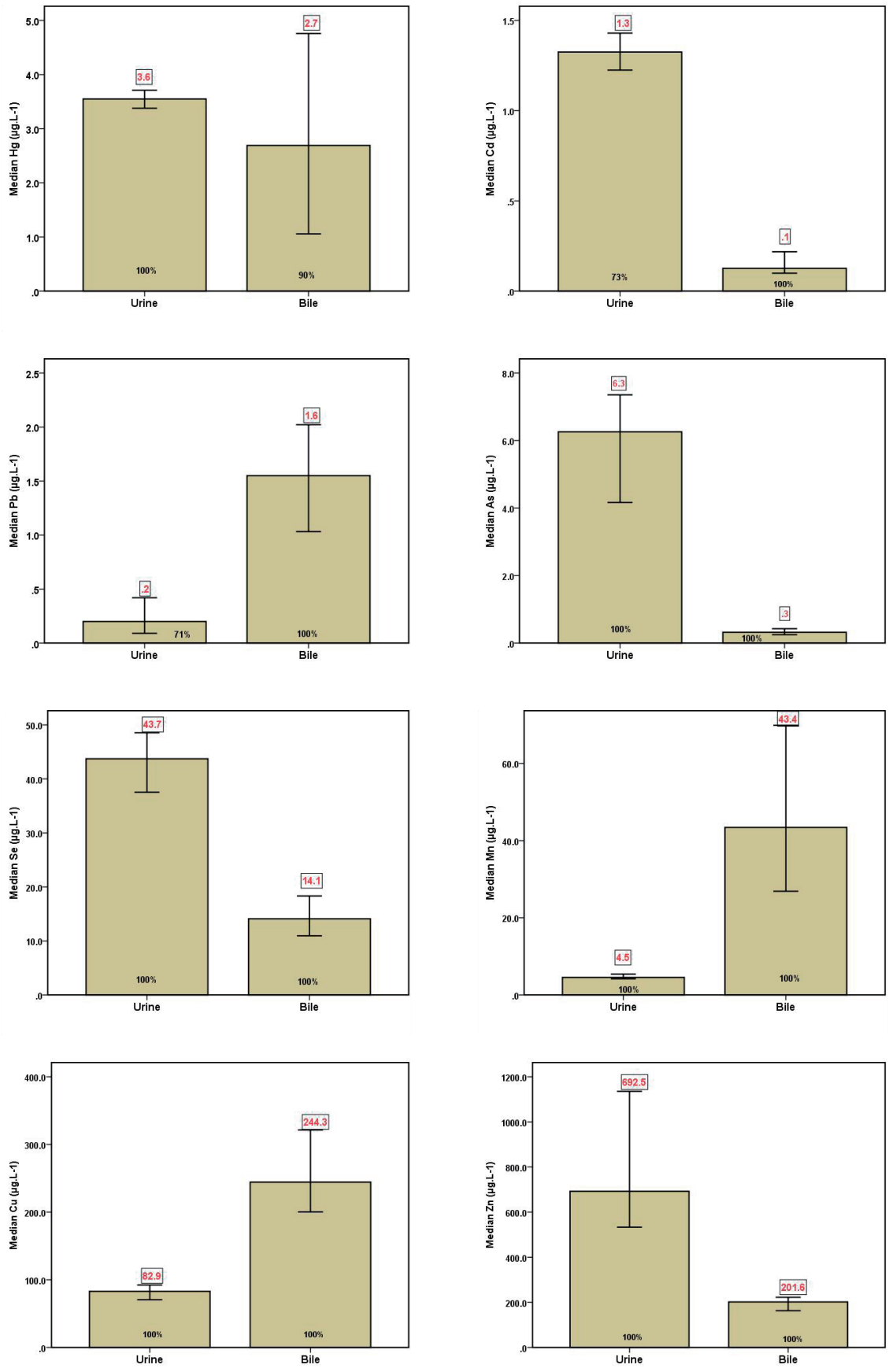

Figure 1. The median values of urinary and biliary toxic and essential metals are in red character. The percentage (%) represents value > the MDL for each metal. Error bars represent 95% confidence intervals.

Table 2. Patient characteristics, clinical parameters, and levels of metals in urine and bile according to fibrosis index (FIB-4) evaluated by the student *t*-test*, χ^2-test** and [a] χ^2 with fisher extract.

	<1.3 (Low risk of advanced fibrosis)			>1.3 (High risk of advanced fibrosis)			*P*
Continuous Variables	*N*	Mean	SD	*N*	Mean	SD	
Age (years)	30	43.8	15.1	28	60.2	12.5	< 0.001*
Height (cm)	30	163.6	8.8	28	165.4	10.8	0.476*
Weight (kilograms)	30	77.9	19.5	28	71.7	18.3	0.213*
BMI (Kg/m^2)	30	29.1	6.7	28	26.0	5.7	0.064*
Hg in urine ($\mu g.L^{-1}$)	26	3.6	0.441	24	3.7	0.541	0.298*
Hg in bile ($\mu g.L^{-1}$)	30	6.3	8.6	27	6.4	9.0	0.504*
Cd in urine ($\mu g.L^{-1}$)	26	3.1	10.8	24	1.3	0.764	0.353*
Cd in bile ($\mu g.L^{-1}$)	30	1.7	4.1	27	1.2	4.8	0.628*
Pb in urine ($\mu g.L^{-1}$)	26	0.334	0.795	24	0.900	1.5	0.011*
Pb in bile ($\mu g.L^{-1}$)	30	2.3	3.4	27	1.7	1.0	0.673*
As in urine ($\mu g.L^{-1}$)	26	8.6	7.7	24	5.1	2.3	0.033*
As in bile ($\mu g.L^{-1}$)	30	0.587	0.705	27	0.427	0.511	0.107*
Se in urine ($\mu g.L^{-1}$)	26	42.9	12.5	24	42.2	12.2	0.816*
Se in bile ($\mu g.L^{-1}$)	30	18.2	12.7	27	14.4	7.7	0.242*
Mn in urine ($\mu g.L^{-1}$)	26	5.1	2.8	24	5.0	1.9	0.964*
Mn in bile ($\mu g.L^{-1}$)	30	84.1	69.6	27	36.4	42.8	0.008*
Cu in urine ($\mu g.L^{-1}$)	26	147.0	314.9	24	92.2	51.1	0.582*
Cu in bile ($\mu g.L^{-1}$)	29	540.6	501.8	27	256.6	247.8	0.003*
Zn in urine ($\mu g.L^{-1}$)	26	776.7	639.4	24	1664.2	2022.5	0.003*
Zn in bile ($\mu g.L^{-1}$)	29	208.1	107.0	27	216.5	104.9	0.658*
Categorical variables	*N*			χ^2 (*p-value*)**			
Residence	60			0.455 (0.588)			
Educational level	60			4.4 (0.036)			
Gender	60			3.8 (0.05)			
Smoking	60			1.4 (0.201)			
Choledocholithiasis	60			0.581 (0.446)			
Biliary stricture	60			2.0 (0.492) [a]			
Cholangiocarcinoma	60			2.22 (0.229) [a]			
Pancreatic cancer	60			1.2 (0.344) [a]			
Primary sclerosing cholangitis	60			0.598 (0.671) [a]			
Post-liver transplant-related biliary complications	60			1.0 (0.306)			
Gall bladder cancer	60			0.950 (1.0) [a]			
Cirrhosis	60			4.3 (0.038)			
Chronic hepatitis B infection	60			0.429 (0.605) [a]			
Wilson's disease	60			0.950 (1.0) [a]			
Post liver transplant	60			0.002 (1.0) [a]			
Diabetes Mellitus	60			3.3 (0.067)			
Hypertension	60			2.4 (0.12)			
Coronary heart diseases	60			0.301 (0.665) [a]			
Lung diseases	60			0.95 (1.0) [a]			
Renal diseases	60			0.598 (0.671) [a]			
Hematological diseases	60			0.002 (1.0) [a]			
Pancreatic diseases	60			2.2 (0.229) [a]			
Malignancies	60			5.9 (0.021) [a]			
Lymphoma	60			1.1 (0.483) [a]			

Table 3. | Comparison of adjusted urinary and biliary metals among patients according to FIB4-index using ANCOVA test.

Analyte		Mean ± SE (95% CI)		F-statistics (*df*)	*p*-value
		FIB4-index ≤ 1.3	FIB4-index > 1.3		
Hg	Urine	3.60 ± 1.03 (3.38, 3.82)	3.74 ± 1.03 (3.53, 3.96)	0.776 (1, 49)	0.383
	Bile	1.37 ± 1.78 (0.43, 4.38)	1.49 ± 1.75 (0.48, 4.57)	0.011 (1, 56)	0.918
Cd	Urine[a]	0.350 ± 2.53 (0.054, 2.28)	1.35 ± 2.33 (0.245, 7.49)	1.69 (1, 49)	0.201
	Bile	0.24 ± 1.57 (0.09, 0.59)	0.25 ± 1.55 (0.1, 0.6)	0.005 (1, 56)	0.945
Pb	**Urine**	**0.06 ± 1.63 (0.02, 0.15)**	**0.19 ± 1.59 (0.08, 0.49)**	**3.08 (1, 49)**	**0.086**
	Bile[b]	1.38 ± 1.21 (0.943, 2.03)	1.21 ±1.2 (0.836, 1.74)	0.266 (1, 56)	0.609
As	Urine[c]	6.50 ± 1.17 (4.73, 8.95)	4.53 ± 1.16 (3.35, 6.12)	2.64 (1, 49)	0.112
	Bile	0.36 ± 1.22 (0.24, 0.53)	0.26 ± 1.21 (0.18, 0.38)	1.46 (1, 56)	0.233
Se	Urine[d]	40.21 ± 1.07 (34.81, 46.43)	38.94 ± 1.069 (33.99, 44.57)	0.105 (1, 49)	0.748
	Bile	14.4 ±1.15 (10.82, 19.18)	10.94 ± 1.15 (8.29, 14.43)	1.98 (1, 56)	0.166
Mn	Urine	4.42 ± 1.12 (3.54, 5.53)	4.48 ± 1.11 (3.63, 5.53)	0.007 (1, 49)	0.935
	Bile[a]	**132.82 ± 1.50 (58.91, 299.17)**	**34.50 ± 1.45 (16.46, 72.24)**	**9.66 (1, 56)**	**0.003**
Cu	Urine	75.87 ± 1.2 (52.30, 110.06)	82.93 ± 1.19 (58.38, 117.80)	0.117 (1, 49)	0.734
	Bile	**441.42 ± 1.22 (294.12, 661.82)**	**185.68 ± 1.21 (126.22, 273.14)**	**9.87 (1, 56)**	**0.003**
Zn	Urine	**574.79 ± 1.23 (377.28, 875.68)**	**1011.31 ± 1.22 (679.26, 1504.18)**	**3.69 (1, 49)**	**0.062**
	Bile	181.09 ± 1.14 (138.66, 236.75)	186.23 ± 1.14 (144.32, 240.33)	0.023 (1, 56)	0.879

All ANCOVA models adjusted for age (years), Gender (Male/Female); BMI (kg/m^2); [c] educational level (≤ high school vs. > high school); Patients with cirrhosis (Yes/No); and patients with diabetes (Yes/No)
Further adjustment: [a] smoking (Yes/No); [b] region of living; [c,d] post liver transplant related biliary complications;
df: degree of freedom

4 Discussion

Excretion of metals from the body is mainly via renal and gastrointestinal routes, with the latter being from the liver through the bile and from the pancreas through pancreatic secretions [37]. This study measured toxic (Hg, Cd, Pb, and As) and essential (Se, Mn, Cu, and Zn) metals in 59 bile and 52 urine samples from patients with biliary and liver diseases. We could not find comparable data to aid the interpretation of our results apart from a few experimental or earlier studies.

4.1 Profile of toxic metals in bile and urine

Most of the bile and urine samples found Hg, Cd, Pb, and As. Hg and As were detected in all urine samples, and Cd and Pb were found in 73% and 71% of the patients, respectively. The highest to the lowest rank order of urinary toxic metals according to median values, in μg.L^{-1}, were As (6.3) > Hg (3.6) > Cd (1.3) > Pb (0.2). Our values were 100%, 73.1%, 40.4%, and 51.9% higher than the medians (μg.L^{-1}) of 0.14 (Hg), 0.179 (Cd), 0.32 (Pb), and 5.7 (As), respectively, estimated by the National Health and Nutrition Examination Survey (NHANES, 2015–2016) for adults ≥20 years old [38]. We also compared our results to reference values for metals in urine established by the German Environmental Survey (GerES) and the Canadian Health Measures Survey (CHMS). All our patients had urinary Hg levels above the GerES reference value of 1 μg.L^{-1} set for adults (18–69 years old) with no amalgam. Urinary Hg reflects long-term exposure to elemental and inorganic forms [39], mainly from dental amalgam fillings, fish consumption, and skin-lightening creams, which we reported in our previous studies [40, 41]. Of our patients who had urinary Cd exceeding 0.8 μg.L^{-1}, the GerES reference value for nonsmokers [42], and 52% had measurements >1.3 μg.L^{-1}, the reference value established by the CHMS for the general population (3–79 years old) [43]. Cd in urine reflects long-term

exposure [44], with smoking and diet being the primary sources in the general population [45]. Only 2 and 5 of our patient's urinary levels were above the CHMS reference values of 27 $\mu g.L^{-1}$ (As) and 1.9 $\mu g.L^{-1}$ (Pb), respectively. Excretion of Pb occurs primarily via urine and feces; both have been considered measures of body burden of Pb [46, 47]. The general population is usually exposed to Pb from various sources such as food, air, water, soil, and dust [47]. Unlike the other three toxic metals, total As in urine indicates recent exposure and most arsenic species' main route of excretion [48], whereas hair and nail reflect past exposure owing to their binding to keratin-rich tissues [49]. Arsenic exists in the environment in both inorganic and organic forms, and its inorganic form is classified by the International Agency for Research on Cancer (IARC) as a Group 1 human carcinogen [50]. Food, particularly rice, is the primary source of arsenic exposure in the general population [51]. Rice is a major part of the Saudi diet that not domestically produced and our previous tested total arsenic in 37 brands of imported rice, and all found above the acceptable regulatory limits [34].

Cd, Pb, and As were detected (>MDL) in the bile of all the patients, while Hg was detected in 90%. Hg had the highest median value (2.7 $\mu g.L^{-1}$), followed by Pb (1.6 $\mu g.L^{-1}$), As (0.319 $\mu g.L^{-1}$), and Cd (0.12 $\mu g.L^{-1}$). Only Pb exhibited a high bile/urine ratio, with a median value of 7.3; 31 (60.8%) patients had a value more than fivefold the median, indicating that bile was the predominant route for excreting Pb. Absorbed Pb that is not stored in bone or soft tissues will be eliminated through the kidneys or bile duct into the intestine [52]. The gastrointestinal tract might further reabsorb biliary metals that subsequently become available for re-excretion in the bile, a process called *enterohepatic circulation* [37], which increases an individual's exposure to toxic chemicals that may cause inflammation, apoptosis, and cell death [53]. The high levels of biliary Pb seen in our study might reflect long-term exposure that may cause liver enlargement and activate inflammatory reactions with the characteristics of moderate cholestasis within the bile ducts [54]. An earlier study reported similar findings [6]. Although the median bile/urine ratios of Cd (0.176) and Hg (0.656) were low, they were more than five times the median values in 13 (25.5%) and 5 (9.8%) patients, respectively. A study related the high urinary excretion of Cd relative to biliary excretion in all patients to old age (>60 years) [6]. Physiological variations in the excretion of creatinine and proteins influence Cd excretion in urine [55]. In our study, 20 patients were between the age of 61 and 81 years, of whom 6 had a ratio of bile/urine Cd >fivefold. Our Hg findings are in line with Ishihara and Matsushiro [6]. The median bile/urine ratio of As was very low (0.054), with none of the patients having a ratio >fivefold. Although no study has As species in bile been tested, it has been suggested that As is most likely excreted into bile in trivalent form because of its ability to bind covalently with thiols such as glutathione [56, 57]. Inorganic As is metabolized into methylarsonate and dimethylarsinate, which are rapidly excreted in urine than inorganic form especially the trivalent form which is highly reactive with tissue components [58, 59]. This may explain our study finding of high As levels in urine (6.3 $\mu g.L^{-1}$) compared with bile (0.319 $\mu g.L^{-1}$); though without identifying the arsenic forms our findings need to be interpreted cautiously. Our observation was in contrast to the findings of Ishihara and Matsushiro [6], in which the levels of As in bile were either equal or greater than in urine. The authors related this to the reabsorption of biliary As by the intestine.

We also observed that some risk factors had a role in the urinary levels of certain toxic metals. Age was associated with higher urinary Pb levels, owing to the release of bone Pb into circulation over time [60], but with lower urinary As owing to decreased As methylation capacity [61]. The positive relationship between urinary Cd levels and BMI might be related to the high percentage of our patients who were diabetic (37%). Human and experimental research suggests that Cd plays a role in diabetes [62]. Last, our patients in the central province, mainly from Riyadh (32 out of 37), had higher biliary Pb levels than those from other provinces. Increased urinary Pb levels were reported previously in Saudi mothers and their infants living in Riyadh, the region's largest and most densely populated city [63].

4.2 Profile of essential metals in bile and urine

Although urine is not considered the ideal matrix for assessing the nutritional status of essential metals, it has been used in a few biomonitoring surveys because of its non-invasive nature [64, 65]. Biomonitoring equivalents (BEs) have also been calculated for specific metals, such as Zn and Se, in different matrices to help interpret their status in association with reference dietary intakes and toxicity guidance levels. We used these values to assess the status of essential metals in our patients.

We based our analysis on the CHMS's urinary reference values in $\mu g.L^{-1}$ of 120 (Se), 25 (Cu), and 1100 (Zn), and 0, 3, and 32 of our patients' levels were below these values, respectively [64]. None of our patients had urinary Mn below the reference value (1.07 $\mu g.L^{-1}$) established for the adult population in Northern France [65]. Although Mn is an essential metal, in excess, it can

be neurotoxic [66], and its normal range in urine should be maintained between 1 and 8 μg.L^{-1} [67]. Three patients had urinary Mn >8 μg.L^{-1} in our study. Hays [68] estimated BEs for urinary Se that were associated with inadequate nutritional Se intake (<10 μg.L^{-1}) and excess selenosis (>90–110 μg.L^{-1}). None of our patients were Se deficient or had excess intake. BEs of urinary Zn between 159 and 206 μg.L^{-1} (deficient) and between 439 and 3489 μg.L^{-1} (protective against toxicity) were established by Poddalgoda [69]. According to the authors, urinary Zn is a more reliable exposure indicator than blood due to homeostasis in blood. Two and 39 patients in our study had urinary Zn levels <159 and >439 μg.L^{-1}, respectively. Two patients had urinary Zn >3489 μg.L^{-1}. Zn deficiency or toxicity has been linked with several chronic health conditions, including liver diseases [70-72].

Excess exposure to Cu primarily causes Cu build-up in the liver, leading to its injury, and is usually assessed in 24-hour urine [73], which we did not collect in this study. However, Ullah [74] found that the amount of Cu per liter in the first-morning urine was almost equivalent to the amount of Cu per 24 hours in patients diagnosed with Wilson disease, in which excess Cu is excreted. In our study, the median (GM) urinary Cu in μg.L^{-1} was ~83 (~82), which seems much higher than values reported in Ireland (GM of 5.1) by Rooney [75], in China (median of 9.28) by Pan [76], and in Taiwan (median of 16.87) by Liao [77]. However, our median value compared favorably to values reported in patients with Wilson disease (87 μg.L^{-1}) [78], and our mean (~119 μg.L^{-1}) was lower than the 250–260 μg.L^{-1} reported in viral hepatitis patients [79].

Considerably high levels of essential metals were found in the bile of our patients. The median values (μg.L^{-1}) of these metals in bile were, in ascending order, 14.1 (Se) > 43.4 (Mn) > 201.6 (Zn) > 244.3 (Cu). Only Mn and Cu levels in bile were a fewfold higher than in urine, with median values of 13.8 and 2.8, respectively, indicating that they are eliminated primarily via biliary excretion [80, 81]. The accumulation of both metals in bile has been linked to inherited disorders [82]. Our findings were in line with Ishihara and Matsushiro [6]. Unlike other metals, Cu in bile does not reabsorb [83]. In contrast to Ishihara and Matsushiro [6], urinary Zn levels were approximately fourfold higher than bile levels. Researchers have reported increased urinary Zn excretion in patients with various health conditions [84-86]. The same trend was seen in Se, where urinary Se was threefold higher than biliary. Urine is the main Se excretion route, reflecting its nutritional status [87]. In our patients, urinary Se levels fell within the normal range, reflecting their adequate dietary intake levels, as reported previously [30]. Earlier experimental studies have shown that Se and As facilitate each other's excretion in the bile [88, 89], and the such conjugate was reported in humans [90, 91], suggesting that Se facilitates the excretion of As. This was observed in our study. A strong correlation was seen between urinary levels of Se and As (r > 0.5); this may explain the low As levels we observed in our patients.

We noted an imbalance in the levels of Cu and Zn that has also been reported in inflammatory conditions [92]. The normal ratio of Cu to Zn in adults is nearly 1:1 [93]. Although only one patient had a urinary Cu/Zn ratio >1 in the current study, the biliary ratio was high, with 33 (~57%) patients having a ratio above 1. A similar observation was reported by Gupta [94], who also found that the biliary and tissue Cu/Zn ratios in patients with gallbladder carcinoma were higher than in patients with cholelithiasis. The higher Cu/Zn ratio reflects the low Zn status in patients with gall bladder carcinoma [94] and hepatocellular carcinoma [95]. The ratio has been used to measure liver disease progression [8, 96].

4.3 Excess toxic metals and/or deficiency of essential metals and hepatic fibrosis

Several liver function tests were performed as part of our patients' clinical assessments (albumin, total protein, total bilirubin, ALS, ALT, and ALP). The pattern of these test results can help determine the area of liver damage for differential diagnosis [97]. As shown in **Table 3**, multiple metals measured in bile or urine demonstrated significant negative or positive relationships with several liver function biomarkers. Because our patients were critically ill, many of their liver function parameters were impaired, which might have influenced these findings, leading to inaccurate interpretations. Alterations in liver function biomarkers were considered indirect markers of liver fibrosis, particularly when combined mathematically [98]. These non-invasive markers were commonly used as an alternative to liver biopsy in patients with various liver conditions such as NAFLD and hepatitis B and C virus [99-101]. We used a simple clinical index (FIB-4) to assess hepatic liver fibrosis in our patients based on the recommended cutoff. The FIB-4 index of 28 (48.3%) was above 1.3 [24]. Urinary Pb levels were threefold higher in patients with FIB-4 index >1.3. In contrast to Cave [102], urinary Pb levels in our study were not associated with ALT or AST. In general, research on Pb and liver fibrosis is scarce. Reja [103] showed that increased blood Pb levels were associated with the severity of liver fibrosis, particularly in patients with NAFLD. A similar observation was reported by Chung [104]. We calculated the hepatic steatosis index (HSI) to determine NAFLD cases based on a score ≥36 [105]. Among our patients, 36 (71.3%) had HSI scores ≥36, yet

their urinary Pb levels were not significantly different from those with low HSI scores. An earlier study showed lower biliary excretion of metals in patients with cholelithiasis [106]. Lower biliary Mn levels (<fourfold) were related to a higher score on the FIB-4 index (p = 0.003). The role of Mn in chronic liver disease, or NAFLD, has been recently documented [107] because it is one of the main required components for superoxide dismutase, mainly responsible for scavenging reactive oxygen species [108]. The progression of liver fibrosis was generally associated with lower serum Zn levels in NAFLD patients [10, 26]. Our study demonstrated that higher FIB-4 indexes were seen in patients with elevated urinary Zn levels. Zinc is an essential element for normal body function that can be toxic at higher levels, particularly with Cu deficiency [109]. We found no correlation between the two metals in urine and bile. Biliary Cu decreased more than twofold with higher scores of FIB-4 (p = 0.003). Lan [110] found a significant relationship between Cu deficiency measured in blood and NAFLD severity based on FIB-4 and HSI scores. Stättermayer [111] stated that the association between low hepatic Cu content and hepatic steatosis and hepatocellular injury might be triggered by other pathological mechanisms that need to be explored.

The findings of this study suggest that the difference in the distribution pattern of biliary and urinary toxic and essential metals may contribute to the pathogenesis of liver fibrosis. Further studies are needed to investigate the nutritional deficiency and toxicological effects of some trace metals in preventing and treating liver diseases.

4.4 Study limitations and strengths

Our study has several limitations: (1) the small sample size may have had impact on the statistical power; (2) the patients had multiple co-morbidities, which likely affected the distribution of biliary and urinary metals; (3) no control data were available, because of the invasive nature of bile sampling; (4) a spot urine sample is not as reliable as a 24-h urine sample for representing exposure to metals, as they are influenced by renal clearance; (5) we could not conduct a creatinine-based estimation of metal levels in urine because of the large number of missing results; (6) potential confounding factors such as genetic factors and exposure to metals from diet, water, and lifestyle behaviors were not measured; (7) hepatic fibrosis was assessed using the FIB-4 index rather than liver biopsy, which is the gold standard method for diagnosis; (8) we did not measure urinary metabolites of As and inorganic As in bile, which carry higher health risks; and (9) owing to the small sample size, we were unable to include the use of medications in the statistical analyses because most patients were on multiple medicines.

Despite these limitations, our study is strengthened by the availability of bile samples to measure metals because data on their biliary excretion are scarce. The sampling of bile for research studies remains an invasive procedure.

5 Conclusions

Our study revealed considerable amounts of toxic and essential metals in the urine and bile of patients. Biliary Pb, Mn, and Cu levels exceeded several folds than in urine, indicating that they are eliminated primarily via biliary excretion. A clear imbalance in biliary Cu and Zn levels was seen, with approximately 57% of patients having a Cu/Zn ratio >1. Higher urinary levels of Pb and Zn but lower biliary levels of Mn and Cu were associated with increased liver fibrosis. Our findings suggest that excess or deficiency of certain metals may play a fundamental role in the pathophysiology of hepatic fibrosis, which needs to be explored.

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Credit Author Statement

Hamad Alashgar-Conceptualization, project administration, and funding acquisition; Ali Albenmousa-Resources; Musthafa Peedikayil-Resources; Mahmoud Abougamel-Resources; Abdulrahman Alfadda-Resources; Fahad Alsohaibani-Resources; Khalid Alkahtani-Resources; Bader AlAjlan-Resources; Faisal Abaalkhail-Resources; Iman Al-Saleh-Metals methodology, formal analysis, visualization, and writing the manuscript. All authors read and approved the manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available due to ethical and privacy restrictions but are available from the corresponding author upon reasonable request.

Ethical approval

Each patient signed an informed consent approved by the King Faisal Specialist Hospital and Research Centre Research Ethics Committee.

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Table S1. Inter-metal correlations in (a) urine and (b) bile (in µg.L^{-1}) using Pearson correlation analysis. (Bold values denoted significant relationships)

a. **Urine (*N* = 52)**

		Hg	Cd	Pb	As	Se	Mn	Cu
Cd	*r*	**0.377**						
	p	**0.006**						
Pb	*r*	0.078	−0.125					
	p	0.581	0.379					
As	*r*	**0.305**	0.050	0.106				
	p	**0.028**	0.726	0.456				
Se	*r*	**0.337**	0.102	0.096	**0.514**			
	p	**0.015**	0.472	0.499	< **0.001**			
Mn	*r*	0.177	−0.149	0.207	0.100	**0.442**		
	p	0.210	0.293	0.140	0.482	**0.001**		
Cu	*r*	0.013	0.241	0.089	0.144	**0.314**	0.057	
	p	0.927	0.085	0.529	0.310	**0.023**	0.687	
Zn	*r*	0.042	0.050	0.227	−0.169	**0.308**	**0.307**	−0.074
	p	0.768	0.724	0.106	0.232	**0.026**	**0.027**	0.601

b. **Bile (*N* = 59)**

		Hg	Cd	Pb	As	Se	Mn	Cu
Cd	*r*	−0.033						
	p	0.801						
Pb	*r*	−0.001	0.253					
	p	0.996	0.053					
As	*r*	0.003	**0.308**	**0.564**				
	p	0.984	**0.018**	< **0.001**				
Se	*r*	−0.174	0.115	**0.370**	**0.258**			
	p	0.188	0.385	**0.004**	**0.049**			
Mn	*r*	0.081	0.134	0.254	**0.337**	0.170		
	p	0.543	0.311	0.053	**0.009**	0.199		
Cu	*r*	−0.011	0.080	0.242	0.170	0.006	**0.338**	
	p	0.933	0.552	0.068	0.201	0.965	**0.010**	
Zn	*r*	−0.002	0.153	**0.309***	0.179	**0.461**	0.005	0.133
	p	0.986	0.250	**0.018**	0.179	< **0.001**	0.969	0.319

r: Pearson correlation coefficient; *p*: level of significance

Table S2. | Pearson correlations between the urinary and biliary levels of metals in µg.L^{-1} (Bold values denoted significant relationships)

			Urine							
			Hg	Cd	Pb	As	Se	Mn	Cu	Zn
Bile	Hg	*r*	0.083	−0.156	0.141	−0.001	−0.024	−0.019	0.101	−0.107
		p	0.561	0.273	0.323	0.997	0.867	0.897	0.479	0.455
		N	51	51	51	51	51	51	51	51
	Cd	*r*	−0.239	−0.249	−0.097	−0.052	−0.259	−0.086	0.051	−0.145
		p	0.092	0.078	0.498	0.717	0.067	0.547	0.722	0.309
		N	51	51	51	51	51	51	51	51
	Pb	*r*	−0.047	−0.048	−0.032	0.104	−0.271	**−0.354**	−0.223	**−0.357**
		p	0.743	0.740	0.824	0.466	0.055	**0.011**	0.116	**0.010**
		N	51	51	51	51	51	**51**	51	**51**
	As	*r*	−0.256	**−0.291**	0.010	0.131	−0.244	**−0.355**	−0.060	**−0.471**
		p	0.070	**0.038**	0.943	0.360	0.085	**0.011**	0.674	< **0.001**
		N	51	**51**	51	51	51	**51**	51	**51**
	Se	*r*	−0.033	**0.295**	−0.233	−0.111	−0.034	−0.089	−0.087	−0.268
		p	0.821	**0.036**	0.100	0.438	0.814	0.535	0.542	0.057
		N	51	**51**	51	51	51	51	51	51
	Mn	*r*	−0.072	−0.068	**−0.447**	0.162	−0.037	**−0.336**	0.032	**−0.362**
		p	0.616	0.634	**0.001**	0.257	0.799	**0.016**	0.822	**0.009**
		N	51	51	**51**	51	51	**51**	51	**51**
	Cu	*r*	−0.243	**−0.353**	−0.232	0.154	0.055	−0.091	−0.027	−0.203
		p	0.086	**0.011**	0.101	0.279	0.703	0.525	0.849	0.153
		N	51	**51**	51	51	51	51	51	51
	Zn	*r*	0.076	0.114	−0.211	−0.085	−0.019	−0.247	−0.013	−0.002
		p	0.596	0.425	0.137	0.555	0.893	0.080	0.929	0.987
		N	51	51	51	51	51	51	51	51

r: Pearson correlation coefficient; *p*: level of significance; *N*: number of case

Table S3. Bivariate analyses testing the relationships between urinary and biliary metals and several demographic, clinical parameters, and risk factors using [a] Pearson correlation analysis for continuous variables and [b] Student *t*-test for categorical variables. Bold values are denoted as significant relationships (< 0.05) or marginally significant (< 0.1).

Continuous variables [a]		Urine									Bile								
		Hg	Cd	Pb	As	Se	Mn	Cu	Zn	Cu/Zn	Hg	Cd	Pb	As	Se	Mn	Cu	Zn	Cu/Zn
Age (years)	*r*	−0.156	−0.042	**0.293**	**−0.305**	−0.199	−0.119	**−0.282**	0.176	**−0.300**	−0.148	−0.091	0.133	−0.103	0.089	−0.109	−0.051	0.154	−0.133
	p	0.270	0.768	**0.035**	**0.028**	0.156	0.399	**0.043**	0.211	**0.031**	0.262	0.494	0.315	0.437	0.501	0.413	0.706	0.250	0.321
	N	52	52	**52**	**52**	52	52	**52**	52	**52**	59	59	59	59	59	59	58	58	58
BMI(Kg/m^2)	*r*	**−0.359**	**−0.295**	−0.178	0.160	0.096	−0.081	0.067	−0.186	0.182	−0.027	0.156	−0.146	−0.001	−0.039	0.089	0.129	−0.054	0.147
	p	**0.009**	**0.034**	0.206	0.258	0.499	0.569	0.638	0.186	0.195	0.840	0.237	0.270	0.997	0.769	0.503	0.335	0.685	0.270
	N	**52**	**52**	52	52	52	52	52	52	52	59	59	59	59	59	59	58	58	58
Categorical risk factors [b]		Urine									Bile								
		Hg	Cd	Pb	As	Se	Mn	Cu	Zn	Cu/Zn	Hg	Cd	Pb	As	Se	Mn	Cu	Zn	Cu/Zn
Gender		0.971	0.755	0.352	0.620	0.102	0.418	0.730	**0.006**	0.106	0.126	0.573	0.378	0.869	0.145	0.867	0.703	**0.065**	0.516
Region of living		0.513	0.586	0.730	0.535	0.769	0.983	0.122	0.538	0.686	0.371	0.189	**0.006**	0.252	0.396	0.725	0.420	0.241	0.180
Educational level		0.905	**0.021**	0.308	0.259	0.413	0.425	**0.018**	0.287	**0.023**	0.470	0.914	0.644	0.779	0.150	**0.084**	0.729	0.440	0.453
Smoking		0.902	< **0.001**	0.369	0.544	0.313	0.737	0.786	0.785	0.684	0.215	0.430	0.219	0.292	0.109	**0.091**	0.762	0.113	0.262
Choledocholithiasis		0.702	0.255	0.469	0.675	0.197	0.849	0.236	0.227	0.802	0.744	0.406	0.550	0.413	0.510	0.866	0.295	0.572	0.462
Post-liver transplant-related biliary complications		0.637	0.142	0.210	**0.059**	**0.004**	0.116	0.296	0.556	0.867	0.764	0.246	0.502	0.424	0.155	0.296	0.278	0.597	0.446
Cirrhosis		0.225	0.735	0.508	0.689	0.869	0.948	0.991	0.334	0.393	0.640	0.429	0.130	**0.005**	0.215	0.240	0.796	0.721	0.974
Comorbid illnesses (Diabetes Mellitus)		0.136	0.443	0.664	**0.026**	0.415	0.297	**0.06**	**0.063**	**0.007**	0.112	0.967	0.305	0.500	0.992	0.814	0.212	0.406	0.446
Hypertension		0.909	0.750	0.868	0.380	0.666	0.115	0.106	0.111	**0.018**	0.631	0.697	0.959	0.209	0.578	0.863	0.966	0.653	0.847

r: Pearson correlation coefficient; *p*: level of significance; *N*: number of cases