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Unraveling the impact of chronic exposure to metal pollution through human gallstones

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ABSTRACT

This study aims to explore the impact of chronic metal exposure derived from persistent pollution from mining activity using human gallstones as proxies. The samples were obtained from patients residing in geologically and environmentally contrasting areas in the Province of Huelva, SW Spain, allowing for the evaluation of the regional effect of metal pollution. The study group resides in the Iberian Pyrite Belt characterized by natural and anthropogenic metal pollution from mining activities, whereas the control group resides in the Ossa Morena Zone famous for its natural parks. A total of 68 gallstones were first classified based on their phase composition and structure and subsequently their chemical composition was studied using solution Inductively Coupled Plasma-Mass Spectrometry. The metal concentrations increased in the cholesterol-rich gallstones from pure, to mixed and composite cholesterol stones along with the increasing amount of minor phases, such as bilirubinate, carbonate, and phosphate. These cholesterol stones did not show an evident enrichment tendency. On the contrary, pigment stones, composed of bilirubinate, carbonate, and phosphate phases, were rich in a variety of elements and the regional comparison showed that the pigment stones from the study area were enriched in sulfide-associated metal(loid)s, Mn, Fe, Cu, Zn, Sr, As, Ag, Sb, and Pb with respect to the control group. Inhalation of polluted airborne particulate matter is considered as one of the main exposure routes among the residents of the study area. Additionally, consumption of local water and locally produced food products such as fruit and vegetables and dermal contact may be possible sources of exposure, but no direct connection was observed.

1. Introduction

Chronic exposure to metal(loid) pollution, *e.g.* from metal mining, may have an impact on human health. Metals are known to have an effect in the generation of various pathologies such as certain types of cancer (Basu et al., 2013; Zhao et al., 2014; Núñez et al., 2016), and they may accumulate in body liquids, soft tissue and hard tissues, *e.g.* urine, blood, hair, teeth, *etc.* (Molina-Villalba et al., 2015; Schoof et al., 2016; Asaduzzaman et al., 2017; Zuluaga et al., 2017). However, metal concentrations in urine and blood represent transitory conditions as their formation is a continuous process. Nevertheless, biomineralizations are possible traps for metal accumulation over relatively long time periods. Gallstones form over several years up to decades by precipitating from the oversaturated bile components in the gallbladder and, hence, they serve as long-term records of metal accumulation. Unless trapped in developing gallstones, one way of eliminating metals from human body is through bile discharge (Szentmihályi et al., 2002; Kohlmeier, 2015). Gallstones can be classified by their phase composition and structure into pure, mixed, and composite cholesterol stones, pigment stones and carbonate stones (*e.g.* Parviainen et al., 2016). Previous studies have demonstrated that pigment stones rich in bilirubin salts tend to contain higher amounts of metals than cholesterol-rich gallstones (Suzuki et al., 1975; Zhou et al., 1997; Suhara et al., 1998; Ashok et al., 2003; Palchik and Moroz, 2005; Rautray et al., 2007; Omer, 2011; Sharma et al., 2015; Weerakoon et al., 2015).

The aim of this work is to compare the chemical composition of different types of gallstones from patients residing in geologically contrasting areas in the Province of Huelva in SW Spain, where the study group is exposed to chronic metal pollution derived from mining activities whereas the control group is not under evident metal exposure. This is the first time that a patient-based study is performed at regional scale on environmentally distinct areas to compare quantitative metal abundances among the study and control groups and to link the impact of metal pollution on human gallstone composition.

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2. Materials and methods

2.1. Geological context

The mining districts in the Province of Huelva offer an unique natural laboratory for the study of environmental impact of metal pollution on humans, as the geology of the area has been extensively studied for decades and the sources of metal pollution and possible exposure routes to humans are well-known (Fernández-Caliani, 2008, 2013; Galán et al., 2008; Sánchez España, 2008; Tornos Arroyo, 2008; Martín-Machuca et al., 2010; Madejón et al., 2011; Castillo et al., 2013; Rivera et al., 2016). The Iberian Pyrite Belt (IPB), one of the largest massive sulfide deposits at the global level (Sáez et al., 1999), crops out in the central part of the Province of Huelva. The polymetallic massive sulfide ore deposits are hosted by volcano-sedimentary rocks (Fig. 1A and B). These massive sulfide deposits are characterized by major sulfide minerals, *e.g.* pyrite, sphalerite, galena, and chalcopyrite, together with a wide variety of minor phases (*e.g.* tetrahedrite, tennantite, arsenopyrite, bismuthinite, native Bi) (Marcoux et al., 1996). It is a heavily polluted area with naturally metal-enriched soils and rocks and with important mining activities since over 4000 years ago (Tornos Arroyo, 2008; Nocete et al., 2014). Vast mining activities in the IPB with over hundred old large-

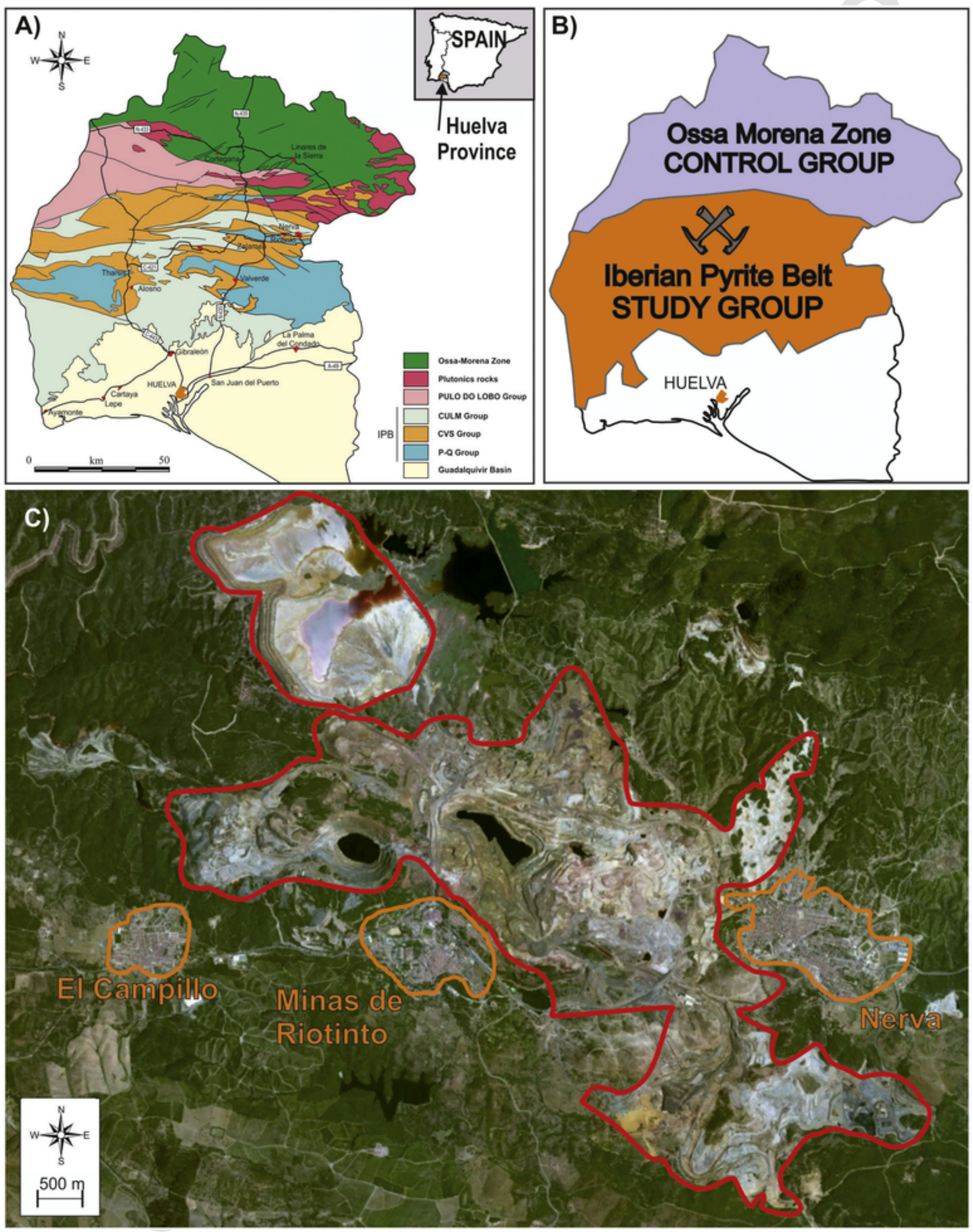

Fig. 1. A) Geological map of the Huelva Province (modified from Almodóvar and Pérez-López, 2008), B) map of the study and control groups based on the lithological context, and C) a satellite map of the footprint of Riotinto mining area indicated with red color and adjacent mining villages indicated with orange color (Google maps) (IPB = Iberian Pyrite Belt; CVS = Volcano Sedimentary Complex; PQ = Shale and Quartzite). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

scale mine workings and several small, ancient workings have been exposed to atmospheric actions (oxidation, meteoric water, and wind) without any remediation measures, thus contributing to the mobilization of metal contaminants and to the pollution risk of the surrounding areas due to generation of acid mine drainage (Fernández-Caliani, 2008). Some of the old mining villages (El Campillo, Minas de Riotinto, Nerva, Tharsis, Valverde del Camino *etc.*) were built in the vicinity of the abandoned mines, and are located to some hundreds of meters from vast open pits and extensive mining waste areas (Fig. 1C).

The village of Valverde del Camino, for instance, is located on the outcropping massive sulfide deposits of the IPB. Dust from the contaminated soils and the mine workings are a direct source of metal pollution by particulate matter (PM) through the atmosphere (Castillo et al., 2013; Fernández-Caliani et al., 2013). Metals may be also transferred from contaminated soils to agricultural plants, and the metal concentrations in the host rocks and soils may affect the groundwater quality of aquifers. These are the main environmental media that serve as sources of metal pollution in the study area. On the contrary, the northern part of the Huelva Province, which is in part occupied by the Natural Park of the Sierra de Aracena and Picos de Aroche, is geologically dominated by complex sequences of granitic, pelitic and carbonate rocks, and constitutes the Ossa Morena Zone (Fig. 1A and B). This area is characterized by high quality groundwater reservoirs and there is virtually no mining (Martín-Machuca et al., 2010; Rivera et al., 2016). Based on this geological context, the patients investigated in this work were divided into the study group residing in the central part of the Province of Huelva characterized by the IPB and the control group residing in the northern part of the province corresponding to the Ossa Morena Zone (Fig. 1B).

2.2. Sample preparation and analysis

The gallstone samples were obtained from the Regional Riotinto Hospital of the Province of Huelva under the informed consent of the patients, and the project has been evaluated positively by the Ethics Committees of the Andalusian Regional Government and by the Spanish National Research Council (CSIC). The hospital receives patients from the central and northern part of the province, and the patients were divided into study and control groups, respectively, based on their place of residence at the time of surgery with the requisite that they have resided at least the past 10 years in the same area. After surgical cholecystectomy, 68 gallstone samples were rinsed with distilled water, dried, and stored at room temperature in plastic cups. Subsequently, the samples were treated and analyzed at the ISO-10000 cleanroom laboratory of the Instituto Andaluz de Ciencias de la Tierra (IACT) and at the Centro de Instrumentación Científica (CIC) at the University of Granada. First, the samples were divided into two halves: one half was ground using an agate mortar with pestle and polished specimens were prepared with the other half. The characterization and classification of all gallstone samples were done as described in Parviainen et al. (2016). More details of the techniques and the criteria for the classification can be found in Parviainen et al. (2016).

Subsequently, bulk element concentrations in the powdered and homogenized samples were determined using acid digestion followed by analysis of solution with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) at IACT and Inductively Coupled Plasma-Optical Emission Spectrometry ICP-OES at the Estación Experimental de Zaidín (EEZ-CSIC) (Granada, Spain). For the bulk analyses, the following sample digestion protocol was developed: 20 mg of the powdered sample (5 mg in case of very small samples) was weighed in Savillex© Teflon vials in the cleanroom laboratory under the metal-free ISO-100 vertical laminar flow hood. The procedure was performed in two cycles in order to assure the total digestion of cholesterol. Each cycle consisted of adding and gently agitating 1 mL of purified HNO_3 (65%) followed by 0.1 mL of H_2O_2 (30%), and placing the closed vials on a hot plate at 100 °C for 24 h. The trace elements (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, As, Ag, Cd, Sb, Pb, U) and Rare Earth Elements (REE; La, Ce, Pr, Nd, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu) were analyzed by ICP-MS, and the major elements (Ca, K, Mg, Na, P, and S) by ICP-OES. For the analyses by ICP-MS, ^{107}Ag, ^{63}Cu, ^{59}Co, ^{56}Fe, ^{55}Mn, and ^{60}Ni were analyzed in He mode, whereas ^{111}Cd and ^{208}Pb were analyzed in no gas mode. For ^{75}As reaction of the beam with O_2 was used to shift the analyte mass to 91 and to eliminate interference mainly by $ArCl^+$ which overlaps with mass 75. Procedural blanks were used to counter possible contributions from the digestion procedure and international reference materials were run to control the quality of the analyses. Standards matching well the gallstone matrix are very limited and attention was paid in the selection of these materials. Bone meal (NIST1486) and limestone (JLs-1), provided by the U.S. National Institute of Standards and Technology (NIST) and Geological Survey of Japan, respectively, were chosen as standards and their compositions analyzed in these runs are similar to certified values (Table 1; except for Na in JLs-1, and Supplementary Table 1). The bone meal represents the organic sample matrix with phosphates, whereas limestone represents the carbonate phases. The limits of detection (LOD) were calculated by summing the mean of the procedural blanks by three times their standard deviation (Table 1 and Supplementary Table 1 for REE).

The patients were interviewed for their living conditions and eating habits with the aim of unraveling the origin of the possible pollution sources. Hence, they were asked for their residence, working background, smoking habit, and origin of the habitually consumed water and food products. This inquiry can be found in the Supplementary Material. The quantitative data from the elemental concentrations and the qualitative data collected in the inquiries were evaluated by statistical methods. Principal Component Analysis (PCA) was performed for the elemental concentrations using XLSTAT-Pro v7.5.2. Basic descriptive statistic, Spearman's correlations and Mann Whitney *U* test for the estimation of the significance of the differences of median values between study and control groups were calculated by using IBM SPSS statistics 23. Significance level was set to $\alpha = 0.05$ unless mentioned otherwise. For the general descriptive statistics of the chemical composition of the gallstones and for the evaluation of differences between groups, median values were preferred over mean because data were not distributed normally; *i.e.* the data were skewed and affected by outliers.

3. Results

3.1. Pure, mixed, and composite cholesterol stones

Cholesterol stones, which can further be classified into pure, mixed, and composite cholesterol stones based on their phase composition and structure (Parviainen et al., 2016), constituted 79% of all the samples. Cholesterol was the main constituting phase, while bilirubinate, different polymorphs of Ca carbonate (aragonite, calcite, and vaterite), and Ca phosphate phases increased from pure to mixed and composite cholesterol stones. Additionally, composite cholesterol stones exhibited a distinct structure with a cholesterol core and an outer layering composed of mixtures of cholesterol with abundant bilirubinate, carbonate, and phosphate. Generally, the elemental concentrations of the three types of cholesterol stones increased in the

Table 1
Results of the certified reference material, NIST 1486 and JLs-1, the accuracy, and the limits of detection (LOD) for the analyzed elements.

Element	NIST 1486	NIST 1486 (measured)	Accuracy (±%)	JLs-1	JLs-1 (measured)	Accuracy (±%)	LOD
Al	*< 1*	BDL	–	110	124	12.7	0.03
Cr	–	–	–	3.37	3.22	− 4.5	0.0001
Mn	1.1	1.2	9.1	13.9	14.6	5.0	4.3E − 05
Fe	99	105	5.6	125	129	3.2	0.006
Co	–	–	–	0.08	0.06	− 25.0	4.9E − 07
Ni	–	–	–	0.36	0.41	13.9	0.001
Cu	*0.08*	0.07	− 12.5	0.27	0.27	0.0	4.9E − 05
Zn	147	119	− 19.0	3.19	3.14	− 1.6	0.005
Rb	0.35	0.33	− 5.7	*0.18*	0.19	4.3	0.02
Sr	264	266	0.8	295	314	6.4	8.7E − 05
As	*0.006*	0.008	33.3	*0.15*	0.15	0.0	3.7E − 05
Ag	–	–	–	*0.0013*	ND		8.5E − 06
Cd	*0.002*	0.002	0.0	0.159	0.157	− 1.3	4.0E − 07
Sb	–	–	–	0.017	0.017	0.0	2.2E − 06
Pb	1.34	1.25	− 6.7	*0.16*[a]	0.12	− 25.0	0.002
U	*0.03*	0.02	− 33.3	1.75	1.78	1.7	1.7E − 06
Ca	265,800	273,303	2.8	393,724	432,915	10.0	0.0001
K	412	442	7.3	24.7	35.8	45.3	0.003
Mg	4660	4116	− 11.7	3655	2855	− 21.9	0.0001
Na	*5000*	6792	35.8	14.4	23.5	63.6	0.0002
P	123,000	126,541	2.9	129	153	19.1	0.002
S	–	–	–	123	153	24.5	0.003

All values are in mg/kg. Italic letters = non-certified elements, ND = not detected.

[a] Mean of the measured values from GeoRem for similar techniques.

following order: pure, mixed, and composite cholesterol stones. Calcium (median 0.15, 1.48, and 4.21 wt%, respectively), K (79.1, 79.2, and 150 mg/kg), Mg (35.2, 136, and 393 mg/kg), Na (819, 979, and 1383 mg/kg), P (527, 761, and 1125 mg/kg), and S (281, 436, and 821 mg/kg) were the most abundant elements (Fig. 2; Table 3). Calcium concentrations increased gradually, partially overlapping among the three types of cholesterol stones and exhibited good positive correlation ($r > 0.690$) with Mg, Mn, Sr, and Pb (Figs. 2–4). Additionally, in the mixed cholesterol stones, Ca correlated well with Fe and Zn ($r > 0.700$). Among the trace elements Mn (1.73, 29.0, and 57.7 mg/kg), Fe (6.46, 28.4, and 26.5 mg/kg), Cu (4.27, 16.7, and 29.3 mg/kg), and Zn (3.67, 6.12, and 11.7 mg/kg) were the most abundant (Fig. 3; Table 2). Rubidium, Sr, Ag, and Pb were present merely in minor concentrations, generally well below 1.00 mg/kg, though Sr exhibited slightly higher concentrations with median values of 2.80 and 6.60 mg/kg in the mixed and composite cholesterol stones, respectively (Fig. 4; Table 2). Chromium (0.10, 0.06, and 0.15 mg/kg), Co (0.003, 0.004, and 0.01 mg/kg), Ni (0.12, 0.10, and 0.10 mg/kg), As (0.005, 0.005, and 0.03 mg/kg), Cd (0.01, 0.005, and 0.01 mg/kg), Sb (0.01, 0.004, and 0.004 mg/kg), and U (0.002, 0.002, and 0.001 mg/kg) exhibited low concentrations or they were not detected in many samples (Supplementary Table 2). Aluminum was not detected in most samples, but exhibited low concentrations in the pure and mixed cholesterol stones (1.47 and 2.24 mg/kg, respectively).

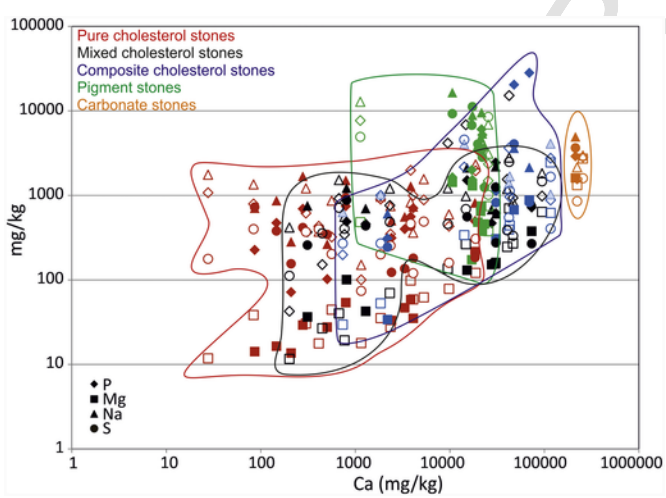

Fig. 2. Concentrations of P, Mg, Na, and S *vs.* concentration of Ca in the different types of gallstones. The colored symbols represent the study group and open symbols represent the control group. (For interpretation of the colors in this figure, the reader is referred to the web version of this article.)

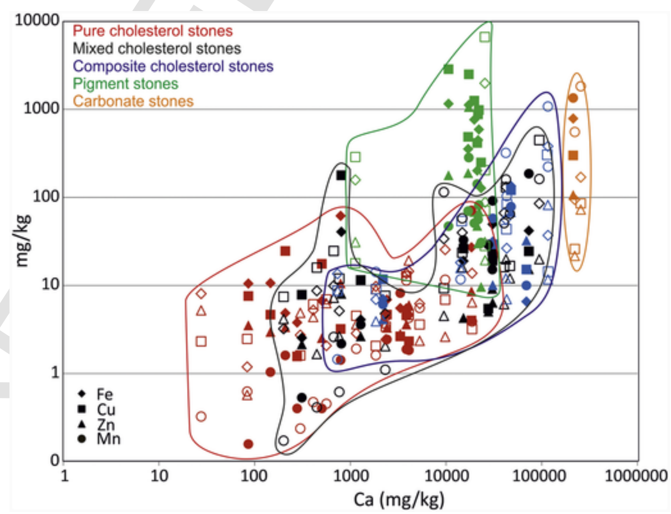

Fig. 3. Concentrations of Fe, Cu, Zn, and Mn *vs.* concentration of Ca in the different types of gallstones. The colored symbols represent the study group and open symbols represent the control group. (For interpretation of the colors in this figure, the reader is referred to the web version of this article.)

Calcium, K, Mg, Na, P, and S exhibited higher median concentrations in the control group than in the study group except for S in pure and composite cholesterol stones and Mg and P in composite choles-

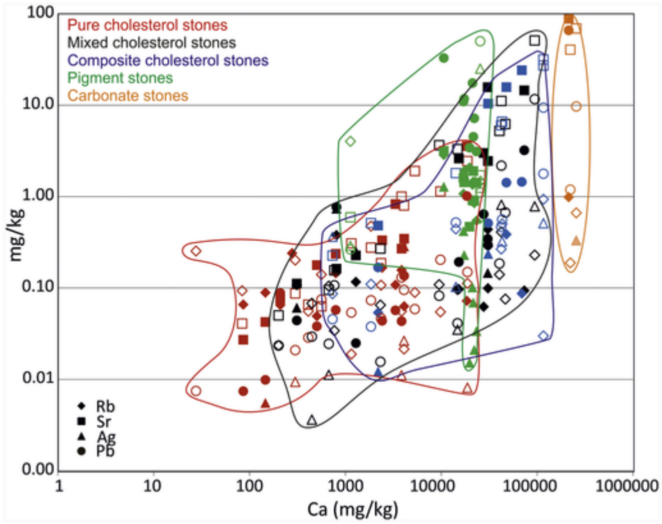

Fig. 4. Concentrations of Rb, Sr, Ag, and Pb *vs.* concentration of Ca in the different types of gallstones. The colored symbols represent the study group and open symbols represent the control group. (For interpretation of the colors in this figure, the reader is referred to the web version of this article.)

terol stones. However, the differences in the median values were not statistically significant. The comparison of the normalized median values of the selected trace elements indicated that Ag, Co, and Sr were more enriched in the study group compared to the control group in the pure and mixed cholesterol stones, in the mixed cholesterol stones, and in the composite cholesterol stones, respectively (Fig. 5). Additionally, Zn, Cd, and Pb were enriched in the study group in the composite cholesterol stones, though these elements exhibited high variation (Fig. 5C). Yet, there is no significant difference between the concentrations of the trace elements between the groups.

3.2. Pigment stones

Pigment stones represented 16% of the gallstone samples, and they were composed principally of bilirubinate, carbonate, and phosphate. Among the different types of gallstones, pigment stones were the most enriched with higher median concentrations of a larger variety of elements in comparison to the other types (Figs. 2–4; Table 2). Calcium (median 2.12 wt%), K (926 mg/kg), Mg (483 mg/kg), Na (6881 mg/kg), P (2000 mg/kg) and S (4046 mg/kg) concentrations were elevated. Manganese (55.9 mg/kg), Fe (446 mg/kg), Cu (876 mg/kg), Zn (47.4 mg/kg), and Pb (7.19 mg/kg) were the most abundant trace elements. On the other hand, Cr (0.50 mg/kg), Co (0.05 mg/kg), Ni (0.21 mg/kg), Rb (1.41 mg/kg), Sr (1.46 mg/kg), As

Table 2
Mean and median elemental concentrations of the different types of gallstones. The values are given in mg/kg unless mentioned otherwise.

mg/kg	Pure (N = 24)		Mixed (N = 19)		Composite (N = 11)		Pigment (N = 11)		Carbonate (N = 3)	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median	Mean	Median
Ca (wt%)	0.34	0.15	2.27	1.48	4.40	4.21	1.89	2.12	23.0	22.2
K	184	79.1	162	79.2	361	150	1339	926	384	434
Mg	49.0	35.2	188	136	566	393	656	483	1885	1608
Na	926	819	1160	979	1953	1383	7531	6881	3347	2969
P	651	527	2029	761	5599	1125	3028	2000	2437	2836
S	318	281	630	436	1345	821	5158	4046	2031	1595
Fe	10.7	6.46	35.4	28.4	65.3	26.5	635	446	350	170
Cu	5.75	4.27	53.1	16.7	63.4	29.3	1523	876	137	85.5
Zn	5.47	3.67	7.84	6.12	23.2	11.7	157	47.4	66.9	72.8
Mn	5.73	1.73	51.4	29.0	173	57.7	78.9	55.9	1243	1345
Sr	0.65	0.27	6.66	2.80	11.3	6.60	1.39	1.41	65.9	69.1
Rb	0.12	0.09	0.14	0.10	0.29	0.27	1.65	1.46	0.61	0.66
Ag	0.06	0.01	0.32	0.15	0.26	0.28	2.81	0.30	0.33	0.33
Pb	0.10	0.06	1.08	0.19	1.49	0.52	13.0	7.19	25.6	9.68
Cr	0.10	0.10	1.08	0.06	1.49	0.15	0.50	0.50	3.88	3.88
Co	0.003	0.003	0.012	0.004	0.02	0.01	0.06	0.05	0.05	0.05
Ni	0.15	0.12	0.73	0.10	0.1	0.10	0.21	0.21	1.87	1.87
As	0.01	0.005	0.01	0.005	0.03	0.03	0.34	0.24	0.06	0.03
Cd	0.006	0.01	0.011	0.005	0.013	0.01	0.22	0.10	0.09	0.09
Sb	0.008	0.01	0.006	0.004	0.006	0.004	0.04	0.03	0.01	0.01
U	0.003	0.002	0.002	0.002	0.004	0.001	0.02	0.01	–	–

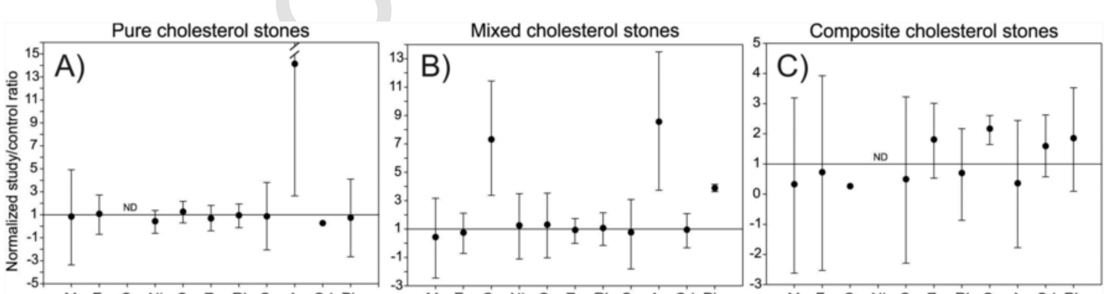

Fig. 5. Normalized median values in the study group with respect to the control group and the error propagations of selected elements for A) pure, B) mixed, and C) composite cholesterol stones (ND = not detected).

(0.24 mg/kg), Ag (0.30 mg/kg), Cd (0.10 mg/kg), Sb (0.03 mg/kg), and U (0.01 mg/kg) appeared in lower concentrations, though generally they were more abundant than in the cholesterol stones. Aluminum was detected only in one sample with 6.60 mg/kg. Most of the trace elements, including Fe, Cu, As, Ag, and Pb, had good positive correlations among each other with *r*-values ranging from 0.707 to 0.917. Zinc, however, showed good correlation with Mn ($r = 0.886$), Sb ($r = 0.704$), and S ($r = 0.690$).

Cobalt, Rb, Sr, Ag, Cd, and S exhibited similar median values both in the study group and control group, whereas Mn, Fe, Cu, Zn, As, Sb, Pb, and U were more enriched in the pigment stones in the study group (Fig. 6A). However, the differences of the median concentrations between the groups were not statistically significant at level $\alpha = 0.05$. One sample from the control group exhibited markedly higher metal(loid) concentrations being an outlier. After excluding the outlier, the normalized graph (Fig. 6B) suggested enrichment of Fe, Cu, Zn, Sr, As, Ag, Sb, and Pb in the study group. In this case, the difference of the median values for As and Pb showed to be significant at level $\alpha = 0.05$ and for Fe and Cu at level $\alpha = 0.09$.

3.3. Carbonate stones

The carbonate stones, composed of different polymorphs of carbonates and minor cholesterol or bilirubinate, are a less frequent type of gallstone represented merely by three samples (one from the study group and two from the control group). They were the major carriers of Ca (mean 22.2 wt%) and Mg (1608 mg/kg), and also were enriched in Cr (3.88 mg/kg), Zn (72.8 mg/kg), Mn (1345 mg/kg), Ni (1.87 mg/kg), Sr (69.1 mg/kg), and Pb (9.68 mg/kg) in comparison to the other types of gallstones (Figs. 2–4; Table 2). Other trace elements, such as Fe (170 mg/kg), Cu (85.5 mg/kg), and S (1595 mg/kg) also exhibited elevated concentrations, whereas Co (0.05 mg/kg), Rb (0.66 mg/kg), As (0.03 mg/kg), Ag (0.33 mg/kg), Cd (0.09 mg/kg), and Sb (0.01 mg/kg) were present in markedly lower concentrations.

A regional comparison between the study and control groups was not feasible for carbonate stones because of the limited database.

3.4. Rare earth elements

The data for the REE indicated that these elements were extremely depleted in human gallstones, and for the most part they were not detected. The general trend was that La (median 4.75 μg/kg) and Ce (4.46 μg/kg) were present at low concentrations in some of the samples rich in cholesterol, but they were below detection limit in the pigment and carbonate stones. On the other hand, Y, Pr, Nd, Gd, Tb, Dy, Ho, Tm, Yb, and Lu were not detected in any samples except for few random cases (Supplementary Table 3).

Because of the small number of samples with detected concentrations of REE, a valuable comparison of the regional differences between the study and control groups was not feasible.

3.5. Socio-demographic description and enquiries

Table 3 summarizes the socio-demographic data and the distribution of gallstone types by gender, age, and group. Female is considered as a risk factor in the gallstone disease (Gallagher and Parks, 2014), which is evident from the gender distribution of the patients: female patients represent 74% (N = 50) and male patients 26% (N = 18) of the total 68 samples investigated in this study. Aging is also a risk factor, and over 65% of the patients were over 50 years old. Generally speaking, the mean age of the patients was 56 years, though there was large variation from 22 to 85 years, especially in case of female patients. Cholesterol stones covered 79% of the samples, followed by 16% of pigment stones and 4% of carbonates stones, and no statistically significant differences were detected between female and male patients in the distribution of the type of calculi.

The samples were distributed in study and control groups in similar portions with 47% (N = 32) and 53% (N = 36), respectively. There was variation in the distribution of the types of gallstones between the study and control group. In the study group the shares of cholesterol stones, pigment stones, and carbonates stones were 72, 25, and 6%, respectively, whereas in the control group the shares were 86, 8, and 6%, respectively. The female patients in the study group presented especially high percentage of pigments stones with 30% in comparison to the control group (4%), whereas in the control group the share of cholesterol stones was elevated with 92% in comparison to the study group (65%), and the differences were statistically significant. Age was also a criterion in the formation of different types of gallstones, with mean ages of 53 and 70 years for patients with cholesterol and pigment stones, respectively. The difference of the mean ages was statistically significant, yet the differences between average ages of each type in the study and control groups, respectively, were not of significance. These data highlighted the higher tendency of formation of pigment stones in the study group, but also that this trend was not owing to the age.

Smoking did not seem to have an impact on the metal(loid) concentrations of the gallstones. Twenty three percent of all of the patients were active smokers, whereas 77% were non-smokers (48% never smoked; 29% ex-smokers). Among the patients with metal-enriched pigment stones, 20% were active smokers, whereas 80% were

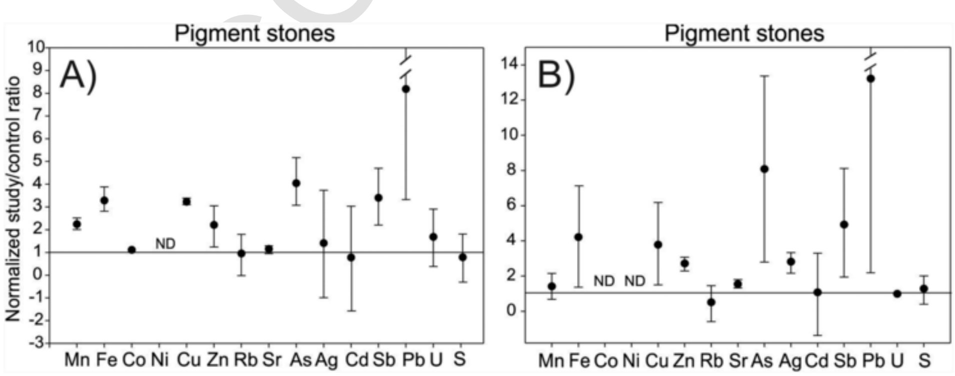

Fig. 6. Normalized median values and the error propagations of selected trace elements of pigment stones A) including the whole data set and B) excluding an outlier in the control group with respect to the study group (ND = not detected).

Table 3
Socio-demographic data and distribution of the different types of gallstones.

	Female	Male	All
Number of samples	50	18	68
% of samples	74	26	100
Mean age (years)	54	60	56
Median age (years)	55	61	56
Min age (years)	22	34	22
Max age (years)	85	79	85
% > 50 years old	61	79	65
Mean age (years) with cholesterol stones	52	55	53
Mean age (years) with pigment stones	69	74	70
Mean age (years) with carbonate stones	51	73	58
% control group	75	25	53
% study group	72	28	47
% cholesterol stones	78	83	79
% pigment stones	16	17	16
% carbonate stones	4	6	4
% chol. stones in control group	92	67	86
% chol. stones in study group	65	89	72
% pigment stones in control group	4	22	8
% pigment stones in study group	30	11	25
% carb. stones in control group	4	11	6
% carb. stones in study group	4	0	3

non-smokers, hence there was no significant difference in comparison to the general trend. Furthermore, there was no correlation between the median metal concentrations in the gallstones and smoking habit. Most of the patients (58% as a whole; 54% and 62% in the study and control group, respectively) consumed water from local origin (tap water from a local reservoir, private well, or a natural spring), whereas 23% (32% and 14%, respectively) claimed to consume merely bottled water (from an unknown spring), and 19% (14% and 24%, respectively) both water sources. All together 77% (68% and 86%, respectively) of the patients reported to consume water from local origin, either partially or exclusively. As for the cooking water, nearly all of the respondents used local water for cooking. Merely, two patients with pigment stones in the study group claimed to consume bottled water for cooking and one patient with cholesterol stones, as well from the study group, used both local and bottled water. Consumption of local food products (local producers or own cultivation) was frequent among the patients (32%; 28% and 35%, respectively), and obtaining the food products from a grocery store was as common (30%; 36% and 24%, respectively). The remaining 38% (36% and 41%, respectively) consumed food from mixed origins, which elevated the partial or total consumption of local products to 70% (64% and 76%, respectively). According to the working background, the patients were divided into five groups: not working (57%; retired, house wife, unemployed, student), service sector (22%; health care, education, cook, cleaning, slaughterhouse), office work (5%; office, bank), industry (10%; mining, blacksmith, construction) and agriculture (5%).

4. Discussion

4.1. Metal(loid) exposure and bioaccessibility to humans

The possible exposure routes of metal(loid)s to human beings include dermal exposure, inhalation, and ingestion. The abundance of metal(loid)s in the IPB is of natural origin, and their mobility has greatly been enhanced by vast mining activities leaving hundreds of square kilometers of Earth's surface strongly impacted by mining wastes (waste rock and tailings), pit lakes, open pits, and old processing plants exposed to weathering conditions without any remediation actions (for instance in Fig. 1C). The risk to human beings residing in the old mining villages lies in the close proximity of the abandoned mining complexes.

Dermal exposure from direct contact with contaminated soil and bedrock is considered as the less important exposure route. For instance, the dermal absorption of As from soil contact is reported to be < 1% (Lowney et al., 2007). However, it cannot be ruled out completely *e.g.* in case of agricultural practices.

Nevertheless, inhalation of dust or airborne PM of geological and anthropogenic origin can be considered as an exposure route that may cause serious health risk for humans. Small PM < 100 μm can stay suspended in the air for a prolonged time being accessible in longer distances from its source. The smaller PM fractions (< 10 μm and < 2.5 μm) may penetrate deeper in the lungs and deposit in the trachea-bronchial or alveolar region, and at the same time, the particles < 2.5 μm are associated with higher toxicity (Oberdörster et al., 2004; Schlesinger et al., 2006; Valavanidis et al., 2008; Zereini et al., 2012). The chemical form of the particles also determines whether the metals are bioaccessible and may cause health risk once deposited in the lungs (Mazinanian et al., 2013). According to Castillo et al. (2013) and Fernández-Caliani et al. (2013), the PM in the windblown dust in the Riotinto mining district preserves the metal pollution signature of the mine wastes, and is a persistent source of air pollution in the area. The airborne PM (< 10 μm) adjacent to the mine waste dumps contains pyrite, chalcopyrite and their secondary phases including *e.g.* goethite, jarosite, and hematite, with elevated concentrations of sulfide-related metal(loid)s (As, Bi, Cd, Cu, Pb, Sb, Zn). *In-vivo* experiments on rats are frequently used in epidemiological and toxicological studies on pulmonary exposure to ambient PM, because rats have a physiological resemblance to humans. These experiments have demonstrated that PM-associated metals (*e.g.* V, Ni, Zn, Mn, and Pb) deposited in the lungs may translocate to systematic circulation and to extrapulmonary organs, such as liver, in which bile fluids are secreted (Wallenborn et al., 2007). Translocation of As is also suggested in a study where rats were subjected to acute inhalation of tailings and waste rock fine PM (Oliaro et al., 2017). Additionally, *in-vitro* studies – simulating metal bioaccessibility in lungs – using contaminated soils and mine tailings, suggested high bioaccessibility for various metal(loid)s (As, Cu, Fe, Mn, Ni, Pb, and Zn; Guney et al., 2017).

Ingestion of contaminated soils is ruled out as this study involves adult patients, and this would come into consideration only in case of small children. Instead, ingestion of possibly contaminated drinking and cooking water and locally produced food products may be possible sources of metals. Contaminated soils and bedrock hosting an aquifer may have an impact on the water quality. Therefore, the consumption of water from local private wells or natural springs is of concern as they are necessarily not subjected to any sanitary controls. Hence, their water quality is not known and may pose a health risk. Likewise, metal pollution from contaminated soil may transfer to edible plants for human consumption, and additionally, the atmospheric deposition may also be a major source of contaminants in both soils and plants (Fernández-Caliani et al., 2013). A recent study on metal emissions (Cd, Cr, Mn, and Pb) from a municipal solid waste incinerator reports that the consumption of local food products (vegetables and cereals) is the major exposure route to humans (Li et al., 2017). Additionally, they recognize inhalation as a possible exposure route due to atmospheric pollution nearby its source. According to another research, high accumulation and translocation of toxic metal(loid)s (As, Cd, Cr, Pb, and Ni) is reported in the edible parts of vegetables (spinach, radish, tomato, chili, and cabbage) growing in agricultural fields contaminated by mixed industrial effluent irrigation (Tiwari et al., 2011). However, there are also studies suggesting that, in plants grown on contaminated soils, metals preferentially accumulate in the roots and aerial parts over edible plant parts (Madejón et al., 2011; Trebolazabala et al., 2017).

The local well and spring waters or the locally produced food products were not analyzed for metal concentrations in this study, yet they were assumed to be potential exposure routes. This assumption considers the translocation of metal(loid)s from water and food products to the intestine and further to other organs. Regardless of the group, majority of the patients consumed drinking water (77%; 68% and 86% in the study and control groups, respectively), cooking water (97%; 94% and 100%, respectively) and food products (70%; 64% and 76%, respectively) of local origin. The remaining 23%, 3%, and 30%, of the patients consumed drinking water, cooking water, and foods of unknown origin, respectively. Regarding the consumption behavior of water and food, the differences between groups were not statistically significant suggesting the similar settings for possible metal exposure. The comparison of median values of the trace elements of interest in the pigment stones in the study group, however, showed persistent higher values for patients consuming non-local drinking water (*i.e.* bottled water) than for patients with local consumption. Yet, only Co, Zn, Cd, P, and S exhibited statistically higher median values. The same comparison for cholesterol stones in the study group, showed the higher enrichment of trace elements in patients consuming bottled water, though merely Rb was statistically significantly enriched. Comparison of the median values of trace elements between samples from patients consuming food products of local *vs.* unknown origin for patients with pigment stones and cholesterol stones, respectively, showed that there was no significant difference either in study or in control group. The results of the regional comparison regarding the origin of the water consumption were unexpected. It is important to highlight that patients are often reticent to fully disclose the nature of their behavior or are less likely to participate in community surveys. Due to their medical condition, patients may be sensitized to claim certain behaviors. Hence, although valuable, survey-based studies can be potentially influenced by biases of the results.

It was considered that the patients with working background in industry (*e.g.* mine workers) and agriculture had the highest risk of metal exposure due to their profession mainly through inhalation or dermal contact. The gallstones (pure and mixed cholesterol stones) of these patients tended to exhibit higher than median concentrations of Mn, Fe, Cu, Zn, and Pb (Cr, Rb, and Sr in some cases), as well as of S in case of the two mine workers in the study group, but the concentrations were not necessarily the highest values. One exception to this trend was a construction worker from the study group having pigment stones with lower than the median concentrations. However, the dependence of the profession with the metal content of the gallstones was not clear as the highest metal concentrations of each gallstone type were recorded in patients who were not professionally active (*i.e.* house wives or retired, N = 3) or who were in cleaning business (N = 1). This implies that the professional background of the patients may have an impact on the metal exposure, but is not decisive.

4.2. Metal(loid) abundances in gallstones: type of calculi vs. regional group

The cholesterol-rich gallstones, namely pure, mixed, and to lesser extent composite cholesterol stones contained lower median values of trace elements in comparison to pigment and carbonate stones. Fig. 5 demonstrates that the trace element concentrations of all cholesterol-rich stones were similar in both the study and control groups and that the elements exhibited large variations. Based on the findings of Parviainen et al. (2017) on the metal distribution in the distinct microphases of the different types of gallstones, cholesterol does not accumulate any trace elements, whereas Ca bilirubinate, Ca Carbonate, and Ca phosphate are the main phases carrying metals. In the cholesterol stones the amount of these metal-carrying phases increased in the order: pure, mixed and composite cholesterol stones. Hence, for this reason the median metal concentrations increased in the respective order (Table 2). This also explains the abundant trace element concentrations (especially Fe, Cu, S, Zn, and Pb) in the pigment stones, which were composed of metal-carrying phases, principally by bilirubinate and also by phosphates and carbonates. The pigment stones were also slightly enriched in Ag, As, Cd, and U with respect to the other type of gallstones (Table 2). When comparing the two groups, Fe, Cu, Zn, Sr, As, Ag, Sb, and Pb were more enriched in the pigment stones in the study group (Fig. 6B), however according to the Mann Whitney *U* test, the differences were statistically significant merely for Fe, Cu, As, and Pb. The characteristic sulfide mineral assemblage of IPB, including *e.g.* pyrite, sphalerite, galena, chalcopyrite, tetrahedrite, tennantite, and arsenopyrite (Marcoux et al., 1996), is in accordance with the pollution signature in the pigment stones. The small sample set of carbonate stones did not allow the comparison of the regional differences, but it can be stated that they were generally enriched in Ca, Mg, Mn, Sr, and to lesser extent to Cr, Ni, and Pb with respect to the other types of gallstones (Table 2). The PCA plotted in the Fig. 7, exhibits the grouping of different types of gallstones and their tendencies with respect to elements corroborating the aforementioned observations. The pure, mixed, and composite cholesterol stones overlapped and some samples (mixed or composite cholesterol stones) exhibited resemblance to the pigment or carbonates stones in agreement with their increasing amount of bilirubinate, carbonates, and/or phosphates. The PCA separating the samples in the study and control groups did not show any clear grouping, especially for the cholesterol-rich gallstones, which is also in accordance with the Mann Whitney *U* test, and is not presented here.

In addition to the metal concentrations incorporated into the constituting phases of gallstones, Parviainen et al. (2016) observed micron-scale metal nodules under ESEM-EDS, which were mostly composed of Cu, Fe, Ni, and Zn and included a variety of minor con-

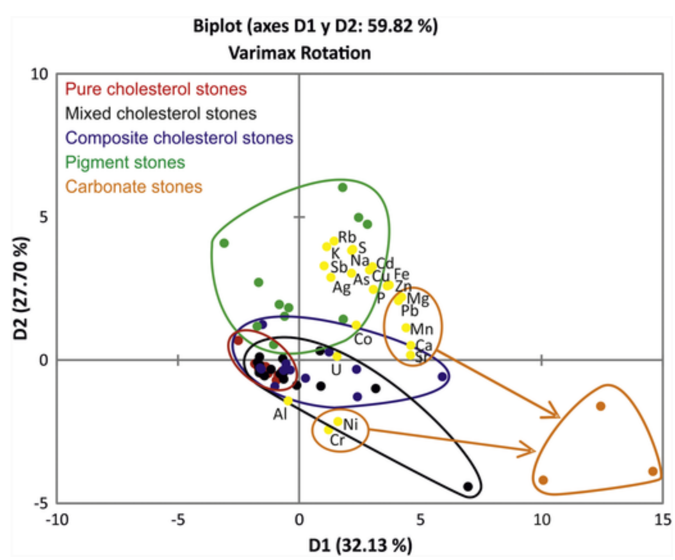

Fig. 7. The PCA for the five types of gallstones and the elemental distribution. (For interpretation of the colors in this figure, the reader is referred to the web version of this article.)

centrations of Al, Ba, K, Mg, Mn, Mo, Sb, Sn, Ti, and S. These metal nodules were observed in all types of gallstones but most frequently in pigment stones and in composite cholesterol stones.

Based on the results of this study, chronic metal exposure among the study group increased the tendency for developing pigment stones. This observation is corroborated by a recent study (Khana et al., 2017) in which Ca, Fe, and Cu concentrations were measured in serum, bile, and gallstones. These authors reported that elevated Fe and Cu concentrations in bile act as promoters for the formation of cholesterol, mixed, and pigmented gallstones. Especially with regarding the pigment stone formation, the bile Fe and Cu concentrations were significantly elevated suggesting that these transition metals promote their formation. Furthermore, the current study shows that the pigment stones exhibited the highest concentrations of a variety of elements. Hence, they are considered as best-suited calculi for environmental proxies.

4.3. Impact of chronic metal pollution on humans

This study revealed that sulfide-related metal(loid)s (Fe, Cu, Zn, Sr, As, Ag, Sb, and Pb) were enriched in the pigment stones (Fig. 6) in patients residing in the study area in close vicinity to outcropping massive sulfide deposits and to abandoned mining activity which has left a huge footprint in the landscape (Fig. 1C). These metal(loid)s are abundant in the massive sulfide deposits of the IPB, and environmental pollution concerning them is frequently described in soil, sediment, water, and atmosphere media (Castillo et al., 2013; Fernández-Caliani et al., 2013; Nieto et al., 2013; Cruz-Hernández et al., 2016). A recent study also corroborates the Pb exposure to humans derived from IPB by investigating the Pb concentrations and isotopes in human hair in residents of a mining village in the same study area as considered here (Zuluaga et al., 2017).

Gallstone disease is a relatively common disease, estimated to affect about 10–15% of the Western World population at some point of their lives (Stinton et al., 2010; Gallagher and Parks, 2014). Chronic exposure to metal pollution may not necessarily increase the rate of occurrence of the disease (though it was not in the scope of this study), but it seems to have an impact on the type of gallstones developed, namely increasing the rate of pigment stones. Similar conditions of exposure to pollution can be assumed for all residents of the same mining village since they breathe the same air and consume the same local water and food products (in reported cases and assumed similar cases). The development of the disease is due to other risk factors (*e.g.* age, gender, pregnancy, diet, obesity, bacterial infections). The difference is that, the residents with gallstone disease preserve the pollution signature in their gallstones in comparison to the residents who are not affected by gallstones and eliminate the metals from gallbladder through bile discharge into the intestine and feces. Therefore, we assume that the risk of chronic metal exposure can be extrapolated to the whole population residing in close vicinity of mining activity in the study area.

5. Conclusions

A patient-based investigation using study and control groups residing in geologically and environmentally contrasting areas was performed in order to evaluate the impact of chronic metal exposure on the gallstone formation and their chemical composition. The study group resides at the IPB, famous for its centuries-old mining activity in Huelva Province, whereas the control group resides at Ossa Morena Zone, northern part of the province.

- This study elucidated that cholesterol-rich gallstones contained lower metal(loid) levels which increased with the increasing amount of metal-bearing phases such as bilirubinate, carbonate, and phosphate, while cholesterol is void of metal(loid)s. Based on the phase-bound control over the metal accumulation, cholesterol-rich gallstones are not suitable as environmental proxies. On the contrary, pigment stones exhibited the highest concentrations of a variety of elements as they were principally formed by bilirubinate and by minor phosphate and carbonate making them best candidates for environmental proxies.
- The chronic metal exposure among the patients in the study group increased the tendency for developing pigment stones.
- Furthermore, the pigment stones in the study group were enriched in metal(loid)s associated to sulfide minerals and host rocks from IPB (Fe, Cu, Zn, Sr, As, Ag, Sb, and Pb).
- The chronic metal exposure in the study group residing in the vicinity of abandoned mining activity is principally attributed to the documented pollution through airborne PM *via* inhalation. Additionally, possible exposure through local water for human consumption and locally produced foods *via* ingestion cannot be completely ruled out. Further, dermal exposure cannot be excluded either due to, for instance, agricultural practices or certain working background though, is considered as less important exposure route.
- Even though pigment stones are suggested as the best proxies among different types of gallstones, one must bear in mind the disadvantages of this type of samples. Obtaining gallstone samples is restricted as it is dependent on aleatory patient illness by cholelitiasis and patient consent whether the samples can be used for scientific purposes, hence, they are not suitable for monitoring or routine use.
- However, we conclude that the results of this study suggested the impact of metal(loid) pollution on the higher rate of formation of pigment stones and metal(loid) enrichment in them in the study group.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.12.224.

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