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Combined microstructural and mineralogical phase characterization of gallstones in a patient-based study in SW Spain - Implications for environmental contamination in their formation

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ABSTRACT

This study explores the environmental impact of metal exposure on humans through detailed phase and structural characterization of gallstones from two environmentally contrasting populations in Huelva Province (SW Spain). A total of 42 gallstone samples, obtained after surgical intervention at the Riotinto Hospital, were studied by powder X-ray diffraction (XRD), Fourier Transformed Infra-Red spectroscopy (FTIR), FTIR-μ-ATR (Attenuated Total Reflection) coupled with an optical microscope, and by Environmental Scanning Electron Microscope with Energy Dispersive X-ray Spectroscopy (ESEM-EDS), and subsequently classified according to their phase composition and structure. Additionally, the patients were enquired for their living habits in order to analyze the source of possible exposure to metal contamination.

The gallstones were classified into pure, mixed and composite cholesterol stones, black and brown pigment stones, and carbonate stones. The patients from the study group residing in a region with acknowledged metal contamination of both natural and anthropogenic origin have a higher risk of metal exposure through contaminated soil, particle matter in the air, and consumption of local water and food products. According to our findings, the metal exposure is related to a higher tendency of forming black pigment stones in the study group in comparison to the control group residing in a natural park with nearly pristine environmental conditions. Moreover, the gallstones from the study group showed to contain more abundant metal components, such as Cu, Fe, Ni, and Zn, than those from the control group. To our knowledge this is the first study to examine the regional environmental impact of metal exposure on human gallstones.

1. Introduction

Gallstones are human biomineralizations composed of organic and inorganic phases, which are formed in the biliary tract. Gallstone disease (cholelithiasis) is a common disease that affects relatively large number of people in Western World; for instance, 10–15% of the population in the United States (Stinton et al., 2010). Surgical intervention (cholecystectomy) is nowadays the best treatment method. There is no general and standardized classification system of gallstones, and the proposed classifications in the literature vary in nomenclature and categories according to the geographical location, study methods and whether composition, color and/or structure are considered (Kleiner et al., 2002; Kim et al., 2003; Qiao et al., 2013; Sharma et al., 2015; Cariati, 2015). The principal lines of classification group the gallstones into cholesterol stones, pigment stones and mixed stones. Rarer gallstones include for instance carbonate, phosphate, cystine, protein, and calcium stearate stones (Qiao et al., 2013). A proper classification system would contribute to a better understanding about the underlying pathogenesis of gallstone disease as the composition and structure of the gallstones provide knowledge about the bile conditions and processes triggering their formation.

Understanding the formation of gallstones is crucial in unraveling the intrinsic and extrinsic processes causing them, but there are still many gaps in our knowledge. Several studies have tried to relate the influence of medical conditions, obesity, diet, genetics, gender, race etc. to the gallstone formation, but the process is very complex and there are multiple risk factors (Katsika et al., 2005; Méndez-Sánchez et al., 2007; Wang et al., 2008). Recent studies reveal that metal contamination of both natural and anthropogenic origin have an adverse impact on human health (Tchounwou et al., 2012; Zhao et al., 2014; Bhowmik et al., 2015; Gu et al., 2016; Núñez et al., 2016). Environmental metal contamination probably has an influence on the gallstone

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formation as well, but this aspect has not been studied in detail. The growth rate of gallstones in-vivo is not well-known. There are indications that their formation takes several years with an approximate growth rate of 1 mm/year (Nudelman, 1993), making them good candidates for long-term recording at decadal temporal scale of prevailing metal exposure to humans.

This study aims at exploring the impact of metal contamination on gallstones formation by comparing two environmentally contrasting populations in the Huelva Province, SW Spain. The study population resides in an area with high metal abundance derived from the naturally metal-enriched bedrock and important historical mining activities in the region of the Iberian Pyrite Belt (central part of the Huelva Province), whereas the control population resides in the Natural Park of Sierra de Aracena and Picos de Aroche (northern part of the Huelva Province). This study bases on a detailed phase characterization and classification of gallstones from patients of both populations by using comprehensive spectroscopic and microscopic approaches.

2. Materials and methods

A total of 42 gallstone samples were obtained from patients who underwent cholecystectomy at the Riotinto Hospital in Huelva Province, which receives patients from central and northern part of the province. The patients signed a consent form authorizing the use of the calculi in this research. The samples are treated totally anonymously and the identity or any personal data of the patient are not revealed at any point. The patients were interviewed after surgery and they were asked questions related to their living habits and diet (Supplementary Data). The patients were divided into a study group and a control group according to their place of residence at the time of the surgery with the aim of tracing the impact of environmental contamination on the formation of gallstones. The prerequisite for each group was to have resided in the area at least during the previous ten years. The study group refers to patients living in the central part of the Huelva Province where there is acknowledged natural and anthropogenic metal contamination derived from massive sulfide deposits of Iberian Pyrite Belt and vast sulfide mining activities since the Third Millenium B.C. (Nocete et al., 2014). The control group corresponds to the northern part of Huelva Province, which is a natural park where there is no direct environmental contamination from the ground and the water reservoirs are of good ecological quality (Martín-Machuca et al., 2010). Although, in this area there are some small and isolated vein-type mineralizations, metal mobilization and local impact on soils and water is very restricted. For more information about the geological characteristics and environmental contamination of the Huelva Province the readers are referred to Fernández-Caliani (2008); Galán et al. (2008); Sánchez España (2008); Tornos Arroyo (2008); Madejón et al. (2011); Castillo et al. (2013); Fernández-Caliani et al. (2013), and Rivera et al. (2016).

After surgery, the samples were rinsed with deionized water, let to dry up at room temperature and stored in plastic cups in the dark. First the samples were photographed and visually characterized by color, size, shape, morphology, and amount of calculi. Subsequently, 29 samples that were large enough were divided into subsamples; larger solitary calculi were cut in half and multicalculi samples were subdivided. One half was ground using a pestle agate mortar and a polished epoxy probe (Epofix) was prepared with the other half. The rest of the smaller samples were merely ground. Rough surface samples were collected in case of abundant sample material.

The homogenized and powdered samples were characterized for phase composition using powder X-ray diffraction (XRD) and Fourier Transformed Infra-Red (FTIR) at the analytical facilities of the Instituto Andaluz de Ciencias de la Tierra (IACT). The bulk powder XRD patterns were collected by Cu K_{α} radiation (lambda = 1.5418 Å) using a PANalytical X'Pert PRO diffractometer equipped with a PIXcel detector operating at 45 kV and 40 mA. The measurements were carried out from 4° to 70° (2θ) with a step size of 0.02°. Automatic-variable anti-scatter slits with a constant irradiated length of 10 mm were used for the incident and diffracted beams. The FTIR spectra were collected using a PerkinElmer Spectrum One FTIR spectrometer equipped with a lithiumtantalate ($LiTaO_3$) detector in nitrogen gas atmosphere. The measurements were carried out in the range from 450 to 4000 cm^{-1} with a resolution of 4 cm^{-1} in absorption mode. A total of 100 scans were collected. Pressed KBr pellets were prepared using 150 mg of KBr and 1 mg of sample in a hydraulic press, and a pure KBr pellet was used to run the background correction.

The polished probes were first observed by an optical microscope for an overall examination and imaging. Subsequently, they were studied by FTIR coupled with a JASCO IRT-7100 microscope using Attenuated Total Reflection (ATR) with a diamond crystal at Centro de Instrumentación Científica (CIC) at the University of Granada. The measurements were run in the range from 650 to 4000 cm^{-1} with a resolution of 4 cm^{-1} in absorption mode, and 300 scans were collected. The spectra were collected using Spectra Manager software. Subsequently, the probes were carbon coated and studied using Environmental Scanning Electron Microscope with Energy Dispersive X-ray Spectroscopy (ESEM-EDS) for imaging in back-scattered mode and for chemical composition at CIC. The acceleration voltage was set to 20 kV for the data acquisition. The rough surface samples were also observed under electron microscope.

The FTIR analysis on ground and homogenized samples is frequently used to characterize gallstone composition (Rautray et al., 2007; Omer, 2011; Cavalu et al., 2015; Weerakoon et al., 2015; Cheng et al., 2016). The FTIR-μ-ATR and ESEM analysis on polished probes are essential for the detection of minor phases providing much more detailed information on the mineralogical composition and structural features of the samples than the traditional bulk XRD and FTIR data. The μ-ATR technique is especially effective for recognition of organic phases, while ESEM can distinguish Ca carbonates and Ca phosphates even at trace amounts, which are undetectable by bulk XRD and FTIR.

3. Results

3.1. Sample characterization

The studied gallstones can be divided into three groups: cholesterol stones ($N = 31$), pigment stones ($N = 8$), and carbonate stones ($N = 3$; Fig. 1). According to their composition and structure, cholesterol stones are further divided into pure cholesterol stones (48%), mixed cholesterol stones (26%), and composite cholesterol stones (26%). Pure cholesterol stones are principally composed of cholesterol (Fig. 1a–c), mixed cholesterol stones contain some bilirubin in addition to the major phase cholesterol (Fig. 1d,e), and composite cholesterol stones have a pure cholesterol core covered by a layered outer crust with variable composition (Fig. 1f,g). One multicalculi sample, composed of different calculi varying in shape and size as well as in composition, is classified as composite cholesterol stone (Fig. 1h). On the other hand, pigment stones can be divided into black and brown pigment stones. Black pigment stones are more common, whereas only one patient had a brown pigment stone.

The size and number of the calculi is highly variable (Fig. 1). The size of all the cholesterol stone types ranges from a few millimeters to up to 4 cm in diameter, whereas the amount can vary from solitary calculus to over 100 small calculi. The solitary cholesterol stones pre-

Fig. 1. Classification of gallstones based on phase composition and structure, and pictures of gallstones and their cross-sections representing each sample type; 1 – pure cholesterol stones; 2 – mixed cholesterol stones; 3 – composite cholesterol stones; 4 – pigment stones; and 5 – carbonates stones. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

sent commonly round or ovoid shape, and polyhedron stones are also observed among the multicalculi samples. The color of the cholesterol stones varies according to the type. Pure cholesterol stones have lighter whitish or light yellowish color though one sample presented greenish color, whereas mixed and composite stones exhibit a variety of darker colors ranging from yellow to brown and black. The pigment stones are brittle solitary calculi or they have only few calculi (< 10). They present small size being generally < 0.5 cm in diameter. The black pigment stones exhibit polyhedron or irregular shape (Fig. 1i–k), whereas the only brown pigment stone sample poses a pyramidal shape (Fig. 1l). The carbonate stones (< 0.7 cm) are black or brownish in color, and present two characteristic appearances: ovoid shape with spiky surface and skeletal shape with smooth surface (Fig. 1m–o).

3.2. Composition and structure of the gallstones

3.2.1. Pure cholesterol stones

Pure cholesterol stones are mainly composed of cholesterol, which was detected by XRD and FTIR in the homogenized samples (Figs. 2a and 3c). These stones exhibit radial structure in the cross-section of the calculi, and the cholesterol laths grow from the center of the calculi outwards (Figs. 1a and 3a–d). The XRD patterns present peaks at 14.03, 15.72, 16.29, 17.51, 18.19, 18.55, and 19.23 2θ, whereas the characteristic FTIR bands of cholesterol are at 2935, 2899, 2866, 1467, 1376, 1057, 953, 838, and 799 cm^{-1}. Pure cholesterol stones may contain traces of bilirubin barely observable in the bulk FTIR spectra presenting a slight peak accumulation around 1600 cm^{-1}. The observations by ESEM-EDS revealed that scarce

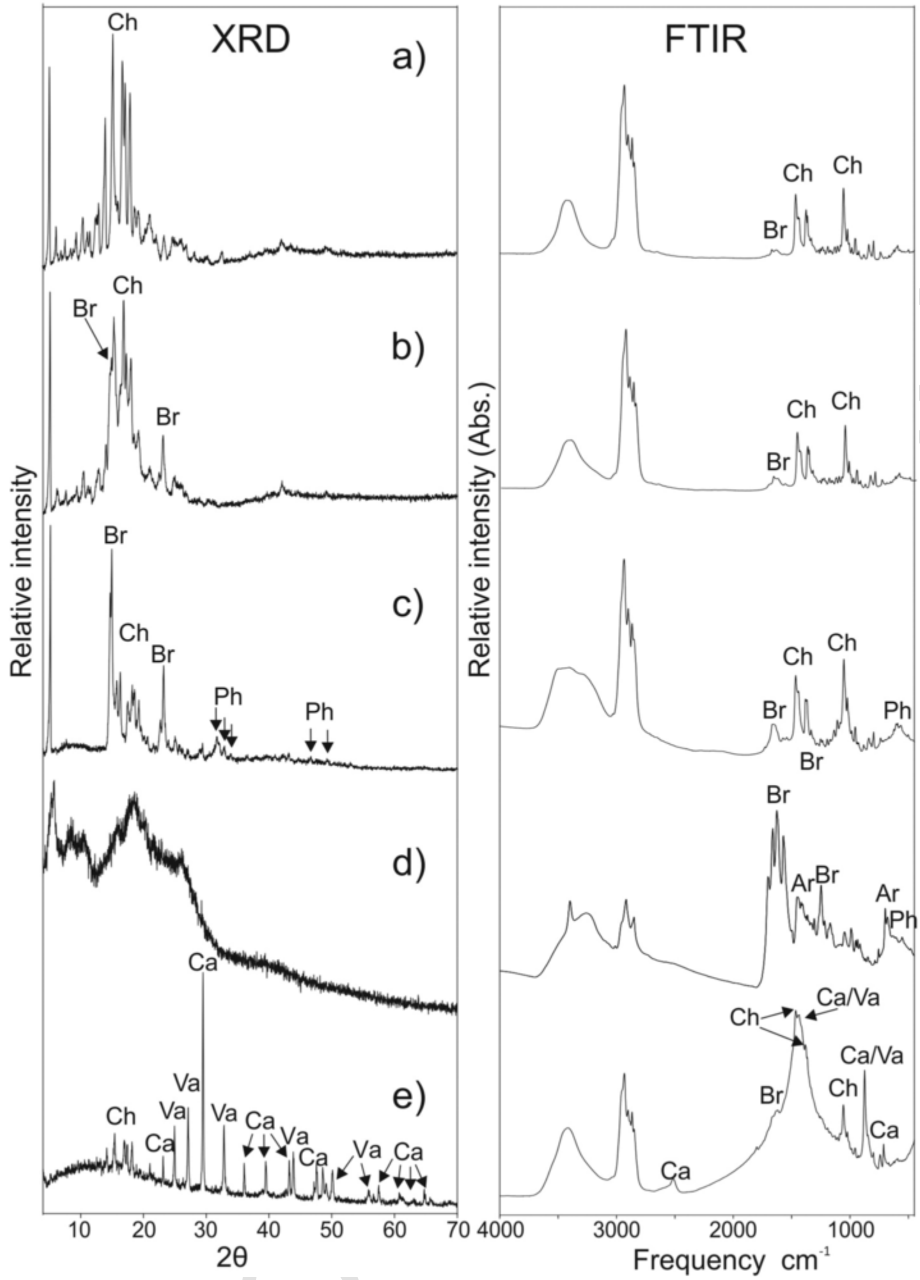

Fig. 2. Representative XRD patterns (left column) and FTIR spectra (right column) of calculi from each classification group presented in Fig. 1; a) pure cholesterol stone (Fig. 1c); b) mixed cholesterol stones (Fig. 1d); c) composite cholesterol stone (Fig. 1g); d) pigment stone (Fig. 1k); and e) carbonates stones (Fig. 1m). (Br = bilirubin; Ca = calcite; Ch = cholesterol; Ph = hydroxyapatite; Va = vaterite).

amounts of Ca carbonate and Ca phosphate are present as round nodules (< 20 μm) and on growth zones, respectively (Fig. 3e,f). In some cases, the carbonate or phosphate crystals are present in the core of the calculi.

3.2.2. Mixed cholesterol stones

Cholesterol is the most abundant phase in the mixed cholesterol stones followed by bilirubin salts (Fig. 2b). Bilirubin was detected by XRD only where it appeared in greater amounts with peak positions at 14.92 and 23.19 2θ (Fig. 2b). These phases commonly appear intergrown and/or bilirubin fills the spaces in between larger cholesterol crystals. The mixed cholesterol stones present concentric layering of cholesterol and bilirubin through the whole diameter of the sample (Fig. 1d). Calcium carbonate and Ca phosphate are minor phases, but they are more abundant—though presenting similar texture—than in the pure cholesterol stones.

3.2.3. Composite cholesterol stones

The composite cholesterol stones have a similar composition to the mixed cholesterol stones; cholesterol and bilirubin are the major phases (Fig. 2c). However, in these type of calculi, the phases are structured in a different manner, and cholesterol tends to form a nearly monomineralic core which is covered by a layered structure of alternating and partly intergrown cholesterol and bilirubin (Figs. 1f,g and 4). In some cases, the composite cholesterol stones present ring-pattern. The presence of Ca bilirubinate in the outer layer of

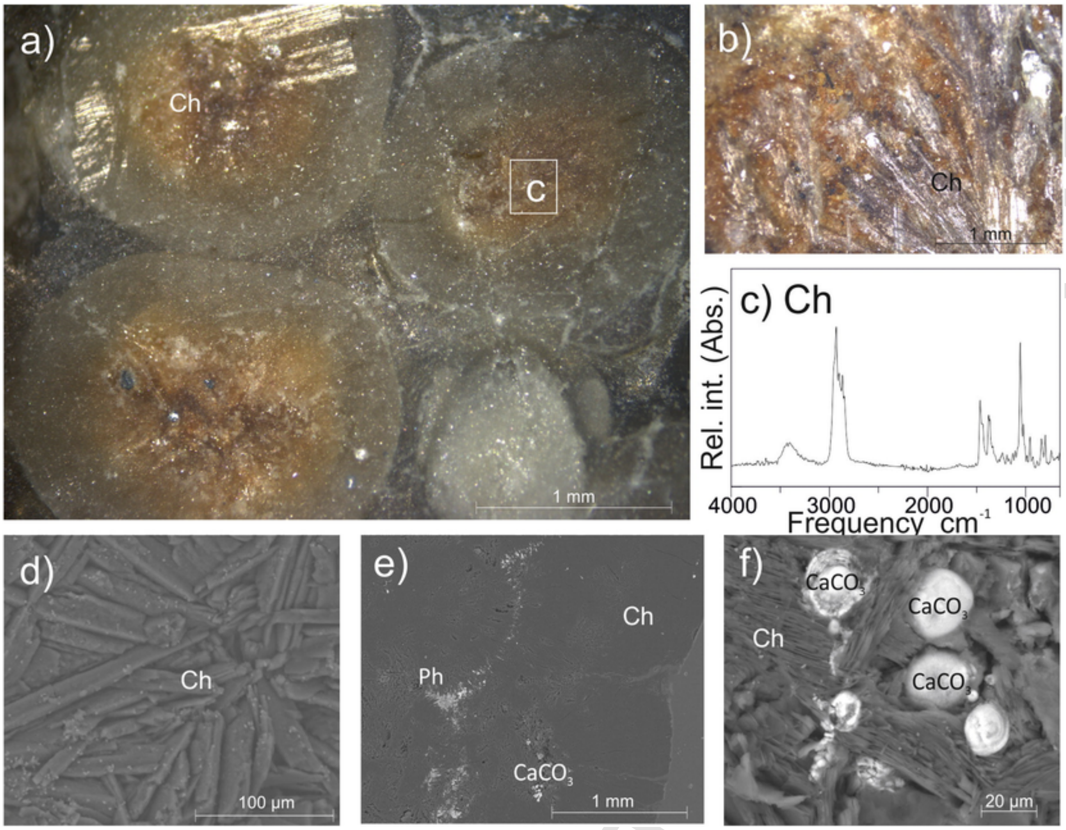

Fig. 3. a) and b) Optical microscopy image of a pure cholesterol stone; c) μ-ATR spectra of cholesterol in the range from 1750 to 650 cm^{-1} of the respective layer indicated in a); d) an optical microscopy image of another pure cholesterol stone; e) and f) back-scattered images presenting details of the same sample. (Ch = cholesterol; Ph = phosphate). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

some composite cholesterol stones was corroborated by ESEM-EDS analysis. This type of cholesterol stone also contains significantly more Ca carbonate and Ca phosphate than the pure and mixed cholesterol stones. The core is characteristically composed of large cholesterol laths (up to 3 mm long) with only scarce precipitations of carbonates and phosphates, whereas they are especially abundant in the outer layers with bilirubin (Fig. 4a–d). In some of the composite cholesterols stones, carbonates and phosphates form even monomineralic layers. The XRD peaks of phosphate of a composite cholesterol stone in Fig. 2c correspond to hydroxyapatite [$Ca_5(PO_4)_3(OH)$], while Fig. 4e and h present the respective monomineralic hydroxyapatite layer with FTIR bands at 2934, 1648, 1416, 1011, 959, and 870 cm^{-1}. The microanalysis revealed that the darker phosphate phase contains traces of Na, whereas the brighter whitish phosphate nodules contain Mg in addition to Na (Fig. 4e). This calculus is covered by a creamy white layer which contains nodules of phosphate (Figs. 1g and 4f). The corresponding FTIR spectrum (Fig. 4i) reveals the presence of cholesterol, phosphate and, possibly, protein, though the peak match was not perfect due to peak overlapping.

3.2.4. Pigment stones

Bilirubin is the major phase in pigment stones in which cholesterol only appears in trace amounts (Figs. 2d and 5). However, typically the pigment stones present a poorly-crystalline character since the XRD patterns show broad diffraction peaks, which makes difficult to perform a detailed characterization (Fig. 2d). Here, the bilirubin FTIR bands are at 3400, 2949, 2924, 2847, 1707, 1666, 1630, 1570, and 1247 cm^{-1} (Fig. 2d). The pigment stones commonly exhibit a layered structure. Carbonates are commonly present as concentric nodules with grain size varying from 5 to 50 μm, and they may also appear as layers. Additionally, abundant phosphates appear as semi-continuous layers and on grain boundaries (Fig. 5d,f). Calcium stearate with band positions at 2915, 2848, 1574, 1537, 1470, 1418, 721, and 665 cm^{-1} is detected in the core of some of the black pigments stones by using μ-ATR (Fig. 5b). The brown pigment stone presents a triangular cross-section in which the layered structure is visible and in the center there is a nucleus of pure cholesterol and Ca-stearate crystals (Fig. 1l). The ESEM-EDS analysis corroborate that the bilirubin salts in pigment stones contain Ca indicating the presence of Ca-bilirubinate (Fig. 5g).

3.2.5. Carbonate stones

Carbonate stones contain different polymorphs of Ca carbonate as major phases, whereas cholesterol and Ca-bilirubinate were identified as minor constituents (Fig. 6a–d). The carbonate stones with spiky surface (Fig. 1m,n) are composed of calcite and vaterite according to the XRD patterns (Fig. 2e), and additionally the ESEM-EDS observations indicated the possible presence of typical crystal forms of aragonite in one of the carbonate stones (Fig. 6c). One spiky stone contains carbonate nodules between cholesterol laths (Figs. 1m and 6b), whereas the other sample has a more compact carbonate crust and in the core the carbonate is present as nodules together with bilirubin (Figs. 1n and 6d). On the other hand, the carbonate stones with skeletal structure (Fig. 1o) are composed of calcite and aragonite.

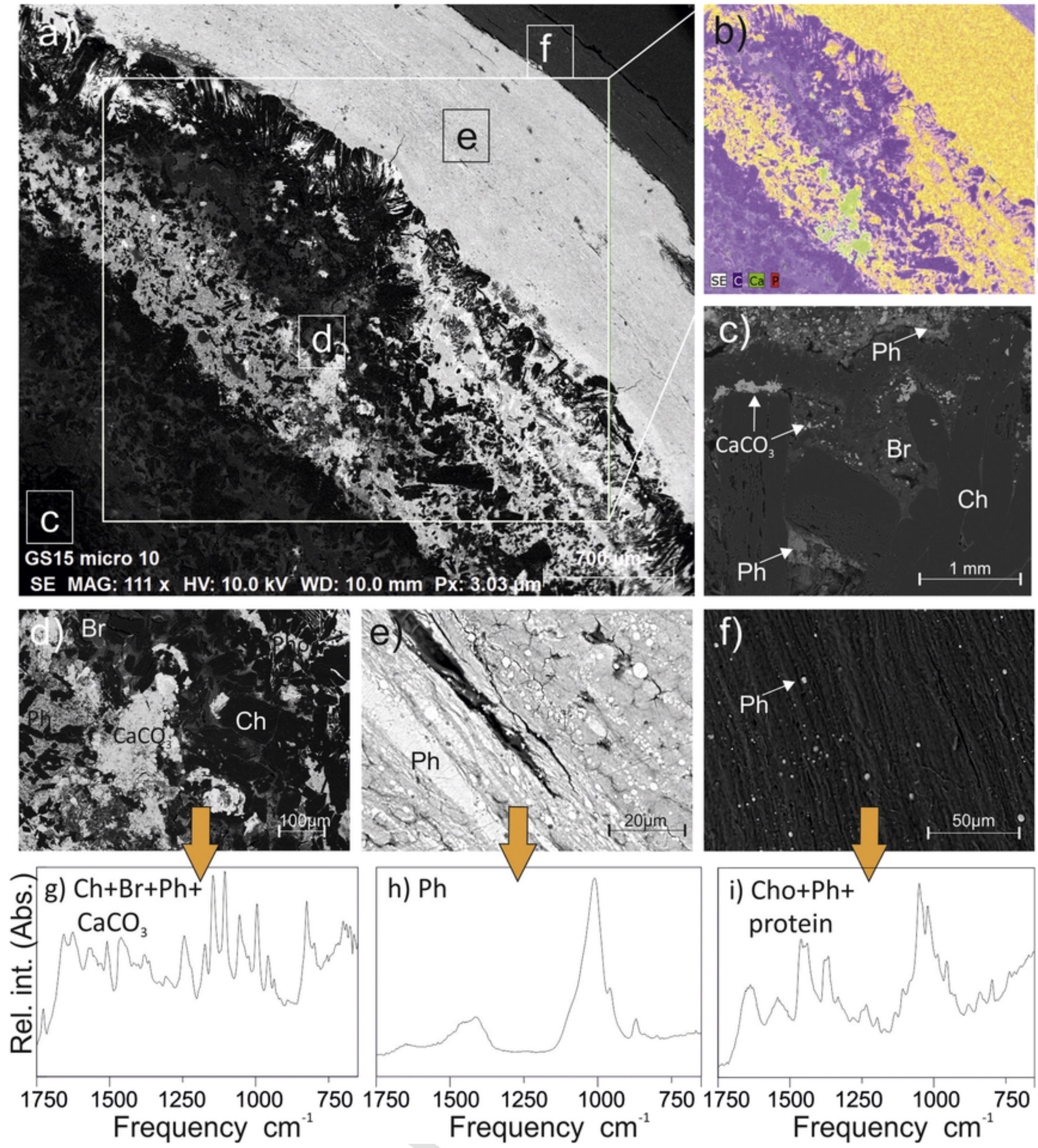

Fig. 4. a) Back-scattered images of a cross-section of a composite cholesterol stone made of a cholesterol core and a phosphate crust (Fig. 1g); b) an elemental map showing the distribution of C, Ca and P; c) a composite cholesterol stone presenting large cholesterol (Ch) laths covered by bilirubin (Br) layers rich in carbonates and phosphates (Fig. 1f); d) a layer of $CaCO_3$ and hydroxyapatite (Ph) appearing together with cholesterol and bilirubin ; e) hydroxyapatite crust with brighter nodules containing Mg; and f) the outermost layer composed of protein and phosphate nodules. The graphs g-**i** represent the µ-ATR spectra in the range from 650 to 1750 cm^{-1} of the respective layers in the figures above (d–f) . (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

3.3. Calcium carbonate polymorphs

Different polymorphs of $CaCO_3$ are common phases in the gallstones, appearing in almost all of the samples, but for the most part only in trace amounts, i.e. only perceptible under exhaustive ESEM examination. Merely 36% of all of the samples contain crystalline carbonates in amounts appreciable in the bulk XRD and FTIR analysis. The pigment stones are more probable to contain $CaCO_3$ polymorphs in high amounts than cholesterol stones (with shares of 80% and 14%, respectively). Generally the pure and mixed cholesterol stones contain very small amounts of carbonates commonly appearing in growth zones and on grain borders (Fig. 3e,f), but also in some cases between the cholesterol crystals. Significant amounts of carbonates appear especially in the layers with abundant bilirubin in the composite cholesterol stones. Calcite and vaterite appear most frequently together, whereas aragonite is preferentially a single polymorph though sometimes it appears together with calcite. All of the three polymorphs were encountered together in one of the carbonates stones (Fig. 6a–c). The ESEM-EDS analysis exhibits that some carbonate phases may contain traces of Mg, Na, and P.

3.4. Minor metal components

The observations by ESEM-EDS revealed small grains—generally < 5 µm in size—with varying metal compositions (Fig. 7). The metal

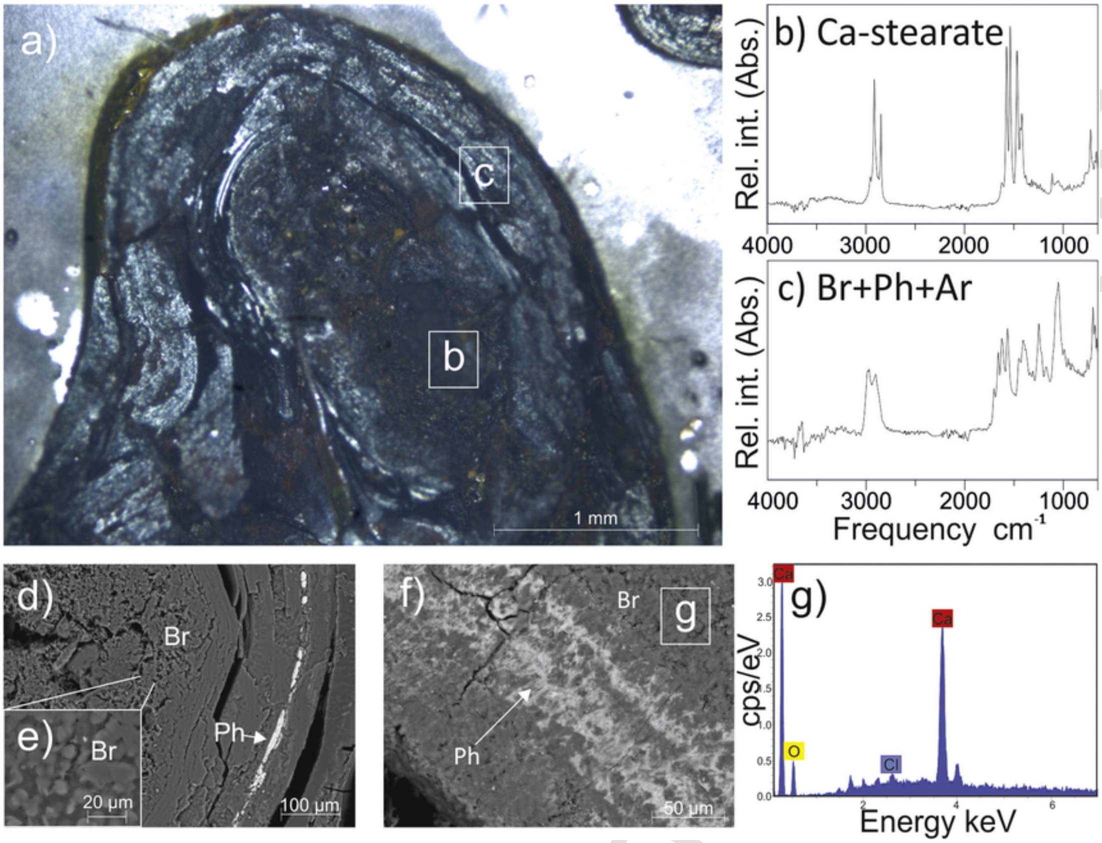

Fig. 5. a) Optical microscopy image of a black pigment stone (Fig. 1j); b) and c) µ-ATR spectra of Ca-stearate, bilirubin, aragonite and phosphate in the range from 1750 to 650 cm^{-1} of the respective layers indicated in a); d) and e) back-scattered images of bilirubin and phosphate layers in a black pigment stone (Fig. 1j) and f) in a brown pigment stone (Fig. 1l); and g) micro elemental spectra by ESEM-EDS exhibiting Ca-concentrations in the brown pigment stone. (Ar = aragonite, Br = bilirubin, Ph = phosphate). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

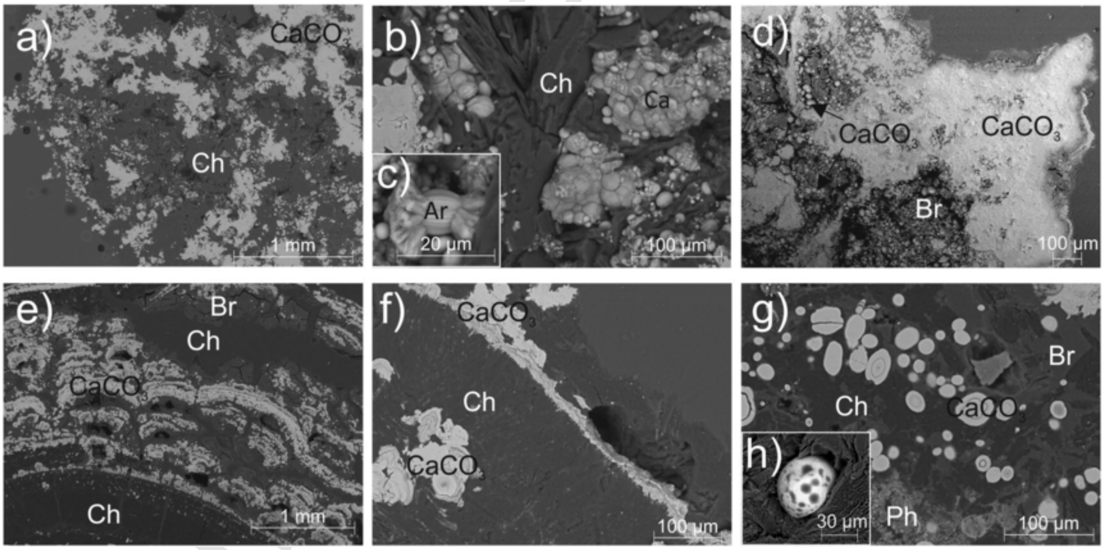

Fig. 6. a), b) and c) Back-scattered images of a carbonate stone (Fig. 1m) presenting calcite nodules and aragonite crystals embedded in cholesterol; d) a carbonate stone (Fig. 1n) presenting a compact carbonate crust and a core composed of carbonate nodules and Ca-bilirubinate e) and f) layer structure of Ca carbonates in composite cholesterol stones; g) and h) concentric Ca carbonate nodules in composite cholesterol stones. (Ar = aragonite, Ca = calcite, Ch = cholesterol, Br = bilirubin, Ph = phosphate).

grains appear in all types of gallstones, though composite cholesterol stones and pigment stones showed to be the most frequent hosts, and they appear in calculi from both groups. The metal composition of these precipitates varies a lot containing abundant divalent cations such as Ca, Cu, Fe, Ni, and Zn. Additionally, minor concentrations of Ag, Al, Ba, K, Mg, Mn, Mo, Na, Sb, Sn, Ti, and W are detected. In

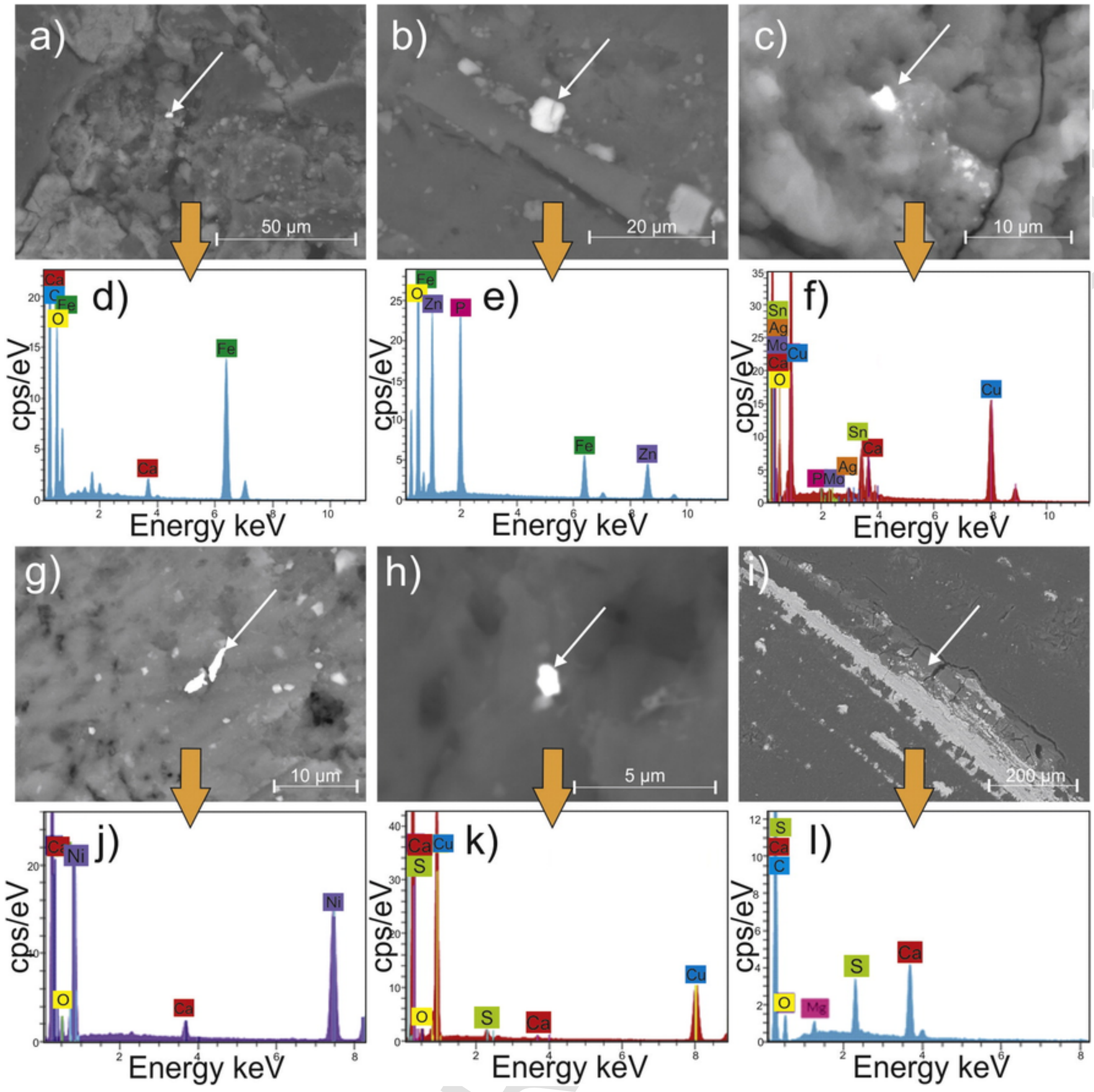

Fig. 7. a), b), and c) Back-scattered images of metal precipitates in two composite cholesterol stones and in one black pigment stone, respectively, from the study group, and their corresponding EDS spectra (d–f); d), h), and i) back-scattered images of metal precipitates in a mixed cholesterol stone, a composite cholesterol stone and in one brown pigment stone, respectively, from the control group, and their corresponding EDS spectra (j–l). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

many cases, small concentrations of P, S or Si are associated with the metals. Iron, Cu, Zn, and Ni (in the relative order of abundance) were the most abundant metals in the calculi from the study group. Iron appears commonly together with Cu or Ca, but also in some grains with Mg and Zn. In one composite cholesterol stone, Fe and Zn appear as a phosphate (Fig. 7b,e). Copper as major metal appears frequently with Ni. The calculi from study group present a wide range of minor metal concentrations such as Al, Ba, K, Mg, Mn, Mo, Sb, Sn, and Ti. In the control group, Fe is the most abundant metal appearing alone or with Ca. Few grains of Cu or Ni appearing with Ca each were also encountered (Fig. 7j and k). One mixed cholesterol stone has a layer made of an unidentified phase with Ca and S (Fig. 7i and l). Zinc was not detected, and the minor metal concentrations detected (Al, K, Mg, Na, Yb) show less variety in the calculi from the control group in comparison to the study group. The precipitates with Ag and W as major metals do not show any clear trend, and these metals are found in one calculus each in both groups.

3.5. Gallstone distribution as a function of socio-demographic and living habits

Gender distribution of the patients revealed that females ($N = 30$, 71%) are more frequently affected by gallstone disease than male ($N = 12$, 29%). Female patients have an average age of 56 years though the age range is wide from 22 to 85 years, and the majority of the patients affected (68%) are over 50 years old. The male age distribution is narrower from 34 to 78 years with an average of 58 years. Similarly, the majority of the male patients (75%) are over 50 years old. Cholesterol stones are the dominant type in both gender groups representing 74% (female 70% and male 83%, respectively) of the samples, while 19% (23% and 8%, respectively) of the samples are pigment stones and 7% (7% and 8%, respectively) correspond to carbonate stones. No statistically significant differences in the distribution of the type of calculi are detected between female and male patients. However, the age is a critical factor in the type of gallstones observed. The average age of the female and male patients with cholesterol stones is 52 and 56 years, respectively, whereas patients with pigment stones present average ages of 67 and 74 years, respectively. The ages of the patients with carbonate stones do not present any trend.

According to the place of residence, 57% ($N = 24$) of the patients belong to the control group, while the remaining 43% ($N = 18$) form the study group. The average age of the patients in the control group is 52 years (standard deviation = 16), whereas in the study group 63 years (standard deviation = 15). There are clear trends in the distribution of the type of calculi between the groups. In the control group, the majority of the patients have cholesterol stones (84%), whereas pigment stones (8%) and carbonate stones (8%) are much rarer with merely two samples of each type. On the contrary, in the study group, pigment stones (35%) are much more common together with cholesterol stones (59%), whereas carbonate stones are less represented (6%) with only one sample. The difference in the portions of patients with pigment stones in each group is statistically significant highlighting the higher tendency of forming pigment stones in the study group.

The two populations have similar smoking habits with 26% of active smokers, 46% of non-smokers, and 29% of ex-smokers (> 5 years since quitting). Regardless of the group, the majority of the patients consume local water for drinking and cooking (tap water originating from local reservoirs, local springs or private well water). Yet, the respondents from the study group consume more frequently bottled water (which origin is considered an unknown spring; 44%) in comparison to the control group (16%). On the other hand, 38% and 53% of the patients in the study group and control group, respectively, consumed local water. The rest with 19% and 31%, respectively, consume both water sources which elevate the intake of partial or total consumption of local water to 57% and 84%, respectively. In case of cooking water, nearly all respondents consume local water except for three patients from the study group who used bottled water for cooking. Similarly, nearly all of the patients obtain the majority of the food products locally. A 34% of the respondents (31% and 37% in the study and control groups, respectively) consume local food products (from a local producer or from own production), whereas 29% (25% and 32%, respectively) obtain the food products from grocery stores (unknown origin of products from supermarket chains). The remaining 37% of the respondents (44% and 32%, respectively) consume products from mixed sources, elevating the intake of partial or total consumption of local food products to 75% and 69%, respectively. Hence, the source of water and food can be considered to be similar in both groups.

Within the patients from the study group there seems to be some important trends. Three of the respondents from the study group with pigment stones had in common the local source of all water consumption (tap water and private well), and all the respondents also affirmed that the majority of the daily food products they consume are of local origin either from a local producer or from own production. On the contrary, two of the respondents with pigment stones consume only bottled water both for drinking and cooking. One of them consumes food products merely from grocery stores, whereas the other from both local and unknown sources.

4. Discussion

4.1. Gallstone formation and their composition

The formation of gallstones is a response to the substances in the bile and interaction of many risk factors such as genetics, female gender, pregnancy, obesity, diet, environmental contamination, and bacterial infections (Stewart et al., 2002; Kosters et al., 2003; Paracha et al., 2012; Gallagher and Parks, 2014). The compositional and textural differences observed in the gallstones in this study indicate that different types of gallstones have distinct mechanism of formation. Moreover, composite cholesterol gallstones exhibit an abrupt change in their composition with a nearly pure cholesterol core and a more bilirubin-rich crust with abundant carbonates and phosphates. The outer layer has a composition with some resemblance to the pigment stones. Cholesterol stones are formed when bile comes supersaturated with cholesterol lipids, whereas pigment stones commonly involve a bacterial infection, and elevated bilirubin secretion i.e. hyperbilirubinbilia is a critical risk factor (Stewart et al., 2002). The changing composition along the cross-section of some gallstones indicates that some patients may evolve different bile conditions during the stone formation. Therefore, the structure of the calculi is an important factor to be taken into account in the classification of the gallstones.

Proteins have been pointed out as important pre-nucleation clusters in the gallstone formation (Binette and Binette, 2000; Sun et al., 2001). This study also highlights that many of the gallstones, regardless of the type, present another phase in their nucleus different from the main phase. The cholesterol stones commonly present Ca carbonate, Ca phosphate or bilirubin in the immediate core of the calculi, whereas some pigment stones present a nucleus of cholesterol or Ca palmitate crystals. Perhaps, randomly floating precipitates in the bile act as nucleation centers in co-assistance with proteins inducing the stone formation. In accordance with this finding, Vítek and Carey (2012) also suggested that brown pigment stones might nucleate around small migratory cholesterol crystals.

Moreover, there are indications that the formation of cholesterol stones is an intermittent and cyclic process (Taylor et al., 1995; Sun et al., 2001; Vítek and Carey, 2012; Cavalu et al., 2015; Qiao et al., 2013). Minor changes in the chemical composition and in the microenvironmental conditions of the bile may induce different patterns of precipitates (Sun et al., 2001). The growth zones with layered precipitations of carbonates or phosphates on the surface of oriented cholesterol crystals or on Ca bilirubinate layers in both cholesterol and pigment stones, respectively, corroborate that there is cyclic tendency in the precipitation of different phases (Figs. 3e, 5d, and 6e,f).

4.2. Relation of metal load and pigment stones

Metal ions are common minor constituents of the bile. Sun et al. (2001) highlighted the importance of the interaction of bile salts and divalent metal ions and their possible role in the formation of pigment stones. Furthermore, previous studies have demonstrated that pigment stones trap higher concentrations of metals than cholesterol stones, and the metals have been claimed to have an impact on the formation of pigments stones (Suzuki et al., 1975; Zhou et al., 1997; Suhara et al., 1998; Ashok et al., 2003; Rautray et al., 2007; Palchik and Moroz, 2005; Omer, 2011; Sharma et al., 2015; Weerakoon et al., 2015). Especially, Ca, Cu, Fe, Mg, Mn, Pb, and Zn concentrations tend to be elevated in the pigment stones. Moreover, Unisa et al. (2011) reported on an elevated risk of gallbladder disease in patients consuming heavy metal polluted drinking water in India. As multiple risk factors influence on the gallstone disease, there is no direct proof of that the metal concentrations in the bile cause the formation of gallstones. Yet, the high tendency of pigment stones rich in Ca-bilirubinate to accumulate elevated concentrations of metals implies that metals play an important role in their formation.

Our study highlights that in a population subjected to environmental metal exposure in a historic sulfide-mining region, i.e. the study group, there is a statistically significantly higher tendency to develop pigment stones in comparison to the control group. Metal precipitates are found in calculi from both groups which was expected. The evaluation of metal composition of the calculi by ESEM-EDS is qualitative, nevertheless, differences can be observed between the groups. The calculi from the study group, especially pigment stones and composite cholesterol stones, contain metallic constituents, such as Cu, Fe, Ni, and Zn with a variety of minor concentrations of Al, Ba, K, Mg, Mn, Mo, Sb, Sn, and Ti. Pigment stones contain also abundant Ca in such phases as Ca-bilirubinate, carbonates and phosphates. In contrast, the calculi from the control group principally contain grains composed of Fe with a lesser variety of accompanying metals. This implies that metal exposure might have an impact on the development of the gallstones and the accumulation of metals in them. The higher frequency of pigment stones in the study group may be also promoted by the environmental metal exposure.

The patients with pigment stones showed to have a higher average age than patients with cholesterol stones, which was also observed in other studies among Chinese and Indian patients (Chandran et al., 2007; Qiao et al., 2013). The average ages of all patients with cholesterol and pigment stones, respectively, present a statistically significant difference. Yet, the difference between average ages in the control and study group is statistically non-significant. Hence, we can deduce that the age is not a factor controlling the higher tendency of formation of pigment stones in the study group. The contamination sources in the study area may be both of natural and anthropogenic origin, and possible metal exposure may occur through several routes, e.g. air, soil contact, consumption of locally produced food such as fruits, vegetables, and meat, and of local water sources, which are surveyed in the patient enquiries. Naturally high metal concentrations in the bedrock and soils are acknowledged in the study area (Tornos Arroyo, 2008). Vast mining has contributed to the mobilization of metal contaminants and multiplied the contamination risk of the surrounding areas as there are several old mining workings exposed to atmospheric actions (oxygen from the air, meteoric water, and wind) without any remediation measures (Fernández-Caliani, 2008). The contaminated soils and the mine workings are a direct source of exposure by particulate matter through the atmosphere (Castillo et al., 2013). Metals may also transfer from contaminated soils to agricultural plants for human consumption, and the metal concentrations in the host rocks and soils of an aquifer may affect the groundwater quality. Through these pathways metals may have an impact on humans. It is assumed that both populations consume similar diet, i.e. Mediterranean diet, yet specific eating habits of each patient are not taken into consideration as the major focus here is the origin of the consumed water and food in the light of the possible contamination sources. In both groups the majority of the patients consume water and food products of local origin, which highlights the similar setting for possible metal exposure. Yet, in one population the metal contamination source is present while absent in the other implying the existing environmental risk factor in the formation of the gallstones in the study group.

Hence, in case of the study group, there is a higher risk of metal exposure through the consumption of local water and food. Additionally, all the patients with black pigments stones reside in old mining villages (one in El Campillo, four in Nerva, and one in Valverde de Camino), which were based in the vicinity of the mines that currently are ceased. These villages are located to some hundreds of meters from vast open pit mines and extensive mining waste areas. Additionally, the village of Valverde de Camino is located directly on the massive sulfide formations of the Iberian Pyrite Belt. The probable exposure routs, hence, may also include contact with contaminated soil and through particle matter in the air (Castillo et al., 2013).

Here we have considered the overall environmental impact of metal contamination through various pathways, and it is challenging to delimit any specific contamination source as major risk factor in the formation of gallstones. However, we can report on metal exposure on humans and clear trends in the type of gallstones formed and their metal components in an area with severe environmental contamination of natural and anthropogenic origin. Additionally, a recent study on environmental exposure to metal pollution in children in Huelva investigated the metal concentrations in urine and scalp hair, and they reported on increased levels of Cd and Mg in urine and As in hair corroborating that environmental metal load in the study area may affect the metal accumulation in human body (Molina-Villalba et al., 2015). This is in agreement with our findings. Ongoing research is being conducted to corroborate our observations by further studying the metal concentrations in the gallstones of a larger population.

5. Conclusions

In the present study, a detailed phase, structural, and compositional characterization of gallstones using XRD, FTIR, FTIR-µ-ATR, and ESEM-EDS provided us a comprehensive assessment of the impact of metal contamination on formation of these human biomineralizations. According to the characterization, the gallstones were classified into pure, mixed and composite cholesterol stones, black and brown pigment stones as well as carbonate stones, each with characteristic appearance.

We report on implications for environmental impact on the formation of gallstones. There are multiple risk factors in the gallstone disease, and according to our results, metal exposure may have also an impact on higher frequency of forming pigment stones. We also observed a higher accumulation of metals in the gallstones, especially in composite cholesterol stones and pigment stones, from the study group under evident environmental metal exposure derived from the naturally metal-rich bedrock and soils of the Iberian Pyrite Belt region and from the historic mining activities. Contaminated soil, and dust in the air, as well as abundant consumption of local water and locally produced food products were recognized as possible exposure routes.

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Appendix A. Supplementary data

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