CONCENTRATION OF MERCURY SPECIES IN HAIR, BLOOD AND URINE OF INDIVIDUALS OCCUPATIONALLY EXPOSED TO GASEOUS ELEMENTAL MERCURY IN ASTURIAS (SPAIN) AND ITS COMPARISON WITH INDIVIDUALS FROM A CONTROL GROUP FORMED BY CLOSE RELATIVES.

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Abstract

Between November 19th, 2012 and December 3rd, 2012, 50 workers were intoxicated with gaseous Hg in San Juan de Nieva (Asturias, Spain) during the maintenance of a heat exchanger of a zinc manufacturer. We have quantified the concentration of methylmercury (MeHg), ethylmercury (EtHg) and Hg(II) in blood, hair and urine samples of those individuals taken three years after the accident. Blood, hair and urine of their closest relatives were also analyzed to assess whether the mercury burden present in the intoxicated individuals was due to the occupational exposure or to environmental or lifestyle-related factors. The determination of the mercury species in the samples was carried out applying multiple spiking Isotope Dilution GC-ICP-MS. This procedure corrects for possible interconversion reactions between the Hg species during the sample preparation procedure. Linear correlations were observed for both groups when plotting MeHg in blood vs MeHg in hair, and MeHg in hair vs Hg (II) in urine. The concentrations of Hg species in the intoxicated individuals were not significantly different from those obtained in the control group except for MeHg in blood. Significantly higher levels of MeHg in blood were obtained in some of the intoxicated individuals who had not consumed fish or seafood since the accident. A different correlation between MeHg in hair and MeHg in blood was obtained for these individuals compared to the control group who showed a hair-to-blood ratio consistent with the reported value for people exposed to Hg via fish consumption. Our results suggest that ingested MeHg followed the same pathway of deposition in hair in exposed and non-exposed individuals. However, the exposed individuals with high MeHg levels in blood showed a significantly different extent of MeHg deposition in hair compared to the control group.

Keywords

Methylmercury; Inorganic mercury; occupational exposure; Hg distribution in humans.

1. INTRODUCTION

Mercury (Hg) is considered by the World Health Organization (WHO) as one of the ten chemicals of major public concern. It is a globally distributed pollutant mostly released to the atmosphere as elemental mercury (Hg^0_v) by anthropogenic activities. Combustion processes, smelting industry or artisanal gold mining increase the human exposure to Hg^0_v specially in developing countries (Baeuml et al., 2011; Dolbec et al., 2000). Other sources of chronical exposure to low doses of Hg are dental amalgams constituted by 50% of Hg^0 (Halbach et al., 2008).

Hg stays in the atmosphere and enters into a dynamic biogeochemical cycle in which it is transformed into different chemical species (Selin, 2009). One of the main risks for humans is the oxidation in the atmosphere from Hg^0 to inorganic mercury (Hg(II)) and its deposition in aquatic ecosystems (Fitzgerald et al., 2007). Hg(II) can be methylated by bacterial activity at the water-sediment interface to form methylmercury (MeHg), which is accumulated by aquatic organisms and biomagnified through the trophic chain (Mason et al., 1995). MeHg is considered the highest neurotoxic form of Hg. Human exposure to MeHg is mostly due to the uptake of fish and seafood. The Minamata tragedy of 1956 revealed the high neurotoxic effects of MeHg among children (Takeuchi et al., 1962).

The increase of Hg emissions into the atmosphere in the last decades has caused a great interest in the biomonitoring of different exposed populations. For this purpose, blood is the preferred bioindicator but due to the complexity of its matrix less invasive bioindicators such as urine or hair are often employed to estimate blood concentrations in large-scale studies (World Health Organization, 2015).

It is known that different Hg species follow a different pathway in the organism (Clarkson, 2002). For example, when Hg^0 enters the bloodstream, it is transformed into Hg(II) by catalase action and

H_2O_2 (Halbach and Clarkson, 1978). Thus, the exposition to Hg^0_v is expected to be reflected by increased levels of Hg(II) in the body. Therefore, total mercury (THg) levels in urine, blood and hair are often used as biomarkers of short-term acute exposure to Hg^0_v (Barregard et al., 2006; Tsuji et al., 2003; Wilhelm et al., 1996). In addition, the long-term exposition to MeHg by contaminated fish uptake has been often evaluated with THg levels in blood and hair (Berglund et al., 2005; Bose-O'Reilly et al., 2010; Budtz-Jørgensen et al., 2004; Dolbec et al., 2000; Drasch et al., 2001). These assumptions are not necessarily true as absorption, metabolism, accumulation, and toxicity of Hg will depend on its chemical form. Also many factors have been found to affect Hg species concentrations in hair, blood or urine (Laffont et al., 2011 ; Laffont et al 2012).

Several studies have shown an increase in blood MeHg after Hg^0_v exposition (Cross et al., 1978; Halbach et al., 2008; Ishihara et al., 1977; Suzuki et al., 1976). Recently, it has been demonstrated that Hg^0_v can be exogenously adsorbed onto hair as Hg(II) (Queipo-Abad et al., 2016). The characteristics of this binding procedure are not well understood yet. Considering the high sulfur content in hair, it is possible that an oxidative adsorption occurs, as it happens with Hg^0_v onto functionalized activated carbon (Korpiel and Vidic, 1997). The external adsorption does not allow the discrimination between exogenous and endogenous Hg (II) coming from the bloodstream. In urine, THg instead of Hg(II) (Berglund et al., 2005; Vahter et al., 2000), has been commonly measured as it has been reported that less than 10% of MeHg is excreted in urine (Clarkson, 2002; Nuttall, 2004). However, several studies showed a correlation between MeHg in blood and Hg(II) in urine, in contrast to the established assumption that Hg(II) in urine comes from Hg (II) in blood (Dock et al., 1994; Rowland et al., 1977; Sherman et al., 2013; Suda and Hirayama, 1992). Thus, the determination of different Hg species in the main biomonitors (blood, hair and urine) can be a valuable tool not only for assessing exposure sources, but also to better understand Hg dynamics and bioaccumulation in humans (Suzuki et al., 1993).

In 2012, several workers were doing maintenance work in a heat exchanger from a zinc manufacturer in Asturias (Spain) and were exposed to high elemental Hg levels (Queipo-Abad et al., 2016). The accident occurred between November 19th and December 3rd, 2012, when some of the workers were taken to hospital with obvious symptoms of acute Hg poisoning. In the following days, it could be concluded that about 50 workers were affected by Hg poisoning according to their high levels of THg in blood. It was concluded that the workers were subjected to elevated gaseous mercury concentrations and that the main exposure route was through inhalation. The evidence of the direct adsorption of Hg in hair for these individuals has been previously reported (Queipo-Abad et al., 2016). In this work, we have measured the concentrations of Hg(II), MeHg and EtHg in blood, hair and urine three years after the accident. Our objective was to find possible chronical exposure biomarkers in the most common human biomonitors. The determination of the Hg species in blood, urine and hair was carried out applying a recently developed procedure based on multiple spiking Isotope Dilution Mass Spectrometry and GC-ICP-MS. This procedure enables the accurate and precise quantification of different Hg compounds while correcting for species interconversions during analysis (Queipo-Abad et al., 2017). We employed this methodology for hair, blood and urine samples of 17 of the intoxicated individuals. This study included also a control group consisting of their closest relatives. The concentrations of the control group were used to assess whether the Hg burden present in the intoxicated individuals, was due to a previous occupational exposure or to environmental or lifestyle-related factors. The applied methodology allowed the simultaneous determination of Hg(II), MeHg and EtHg in each biomonitor. Thus, valuable information about the distribution of Hg species in the organism could be obtained.

2. EXPERIMENTAL SECTION

2.1 Sample collection

We analyzed samples from 17 intoxicated individuals involved in the occupational accident and 10 samples from a control group constituted by the closest relatives of 10 out of 17 intoxicated individuals. The control samples were also collected three years after the accident. For each individual, the three sample matrices, (blood, hair and urine) were collected in the same day. Urine samples were collected in polypropylene tubes and stored at -20ºC until analysis. The hair was cut using stainless steel scissors at the scalp level in the occipital area and stored immediately in two plastic (PE-LD) zip-lock bags. Hair samples longer than 10 cm were cut into two sections to obtain samples of 3-5 cm from the root. Before analysis, hair samples were thoroughly cut into small pieces with scissors to facilitate homogenization and digestion. Blood samples were extracted by qualified personal, introduced in BD Vacutainer® Tubes (K2-EDTA) and stored at -20ºC until analysis.

2.2 Quantification of the samples by triple spike isotope dilution analysis.

The samples were weighed in 10 mL microwave glass vessel. A different sample amount was weighed for each matrix: 0.10 g of hair, 0.15 g of blood or 0.50 g of urine. Immediately, all the samples were spiked with a known amount of ^{201}Hg-enriched MeHg, ^{198}Hg-enriched EtHg and ^{199}Hg-enriched Hg(II). The use of three labelled analogues, each one enriched in a different Hg isotope, allows the correction of species interconversions during the analytical procedure. More details on instrumentation, sample preparation procedure and calculation of concentrations are given in a previous work (Queipo-Abad et al., 2017) and in the Supplementary material. All calculations and statistical analyses were carried out using Microsoft Excel spreadsheet software.

3. RESULTS AND DISCUSSION

3.1 Quality control of the measurements

In each measurement session we analyzed certified reference materials for quality control purposes and procedural blanks to assess possible contamination sources. Level 3 of the Standard Reference Material 955c (*Caprine Blood*) obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) was analyzed for quality control in blood samples. The certified reference material IAEA-086 (Human hair), from the International Atomic Energy Agency (IAEA, Vienna, Austria), was analyzed for quality control of human hair samples. Due to the lack of certified reference materials for urine, (Queipo-Abad et al., 2017) the quality control of urine samples was carried out with the analysis of fortified samples. Table S.1 of the Supplementary material shows that the average results from all analytical sessions obtained for the Level 3 of NIST 955c and for IAEA 086 were in agreement with the certified values.

We have carried out an additional validation with the comparison of total Hg levels in blood with those provided by the Laboratory of Medicine of the Central University Hospital of Asturias (HUCA). Five blood samples from exposed individuals were analyzed in both laboratories. More details on the accredited procedure applied by the HUCA are given in the Supplementary material. Table S.1 shows that the sum of the concentrations obtained in the samples by our speciation methodology (THg = Hg(II) + MeHg + EtHg) were in general agreement with the THg values provided by the HUCA particularly for high Hg levels. For lower total Hg levels, the results obtained by HUCA were slightly lower than our results probably due to poorer limits of detection in the HUCA procedure.

3.2 Hg species concentration in blood, hair and urine samples.

Hg species concentrations in blood, hair and urine of the 17 intoxicated workers were compared with those obtained in the control group. The control group consisted of 10 close relatives of 10 exposed individuals. The level of EtHg in all samples from both groups were below the limit of

165 quantification of the method (0.26 ng Hg g^{-1}) (Queipo-Abad et al., 2017). This result is consistent
166 with the assumption that the only known source of EtHg is vaccines containing thimerosal as
167 antibacterial preservative (Clarkson, 2002). Table 1 shows the individual MeHg and Hg(II) levels
168 measured in the three matrices for the exposed individuals and the control group. The additional
169 information of the individuals is summarized in Table S.2. The descriptive statistics for the
170 concentration results classified using the information given in Table S.2 are shown in Table 2. The
171 same descriptive statistics are used in Table S.3 to show the percentage of the different species in
172 the samples.

173

174 *3.2.1 Hg species concentration in blood*

175 As can be observed in Table 2 we obtained similar geometric means of Hg(II) concentration in the
176 blood samples of both groups (1.2 and 1.5 ng g^{-1}). However, the MeHg geometric mean of the
177 exposed individuals (8.1 ng Hg g^{-1}) was significantly higher than that obtained in the control group
178 (5.8 ng Hg g^{-1}). Indeed, the percentage of MeHg in blood of the exposed individuals ranges from 46
179 to 98 % (Table S.3). The only blood sample in which MeHg is not the major Hg species
180 corresponds to the oldest person of the study (individual 24). Additionally, we obtained surprisingly
181 high concentrations of MeHg in blood in five of the exposed individuals who reported low fish
182 consumption (individuals 4, 5, 7 and 8) or even, no consumption at all (individual 14). Table 2
183 shows that median blood MeHg concentrations increase with fish consumption. However, the
184 geometric mean of MeHg concentration in blood of all individuals (both control and exposed)
185 consuming fish 3 or 4 times per week (6.7 ng Hg g^{-1}) is very similar to that obtained in non-fish
186 consumers (6.3 ng Hg g^{-1}). For example, the MeHg concentration in blood of a non-occupationally
187 exposed individual who eats fish everyday was 9.3 ng Hg g^{-1}, whereas, as observed in Table 1 and
188 Table S.3, other individuals consuming fish 2 times per week presented similar levels (9.1 ng Hg g^{-1}).
189 The highest MeHg concentration in blood (21.6 ng Hg g^{-1}) corresponded to an occupationally

exposed individual who reported a lack of fish consumption since the accident. Taking into account the reported half-life of MeHg in blood (Díez, 2008; Jo et al., 2015), such a high MeHg concentration may not be due to fish consumption. Previous works showed that certain foodstuffs different from fish also contain significant Hg levels (European Food Safety Authority, 2012), such as poultry (Cabañero et al., 2005; Yin et al., 2017). However, Tables 2 and S.4 do not show any clear correlation of MeHg concentration and MeHg percentage with poultry, beef or pork consumption.

3.2.2 Hg species concentration in urine

In the case of urine, there are several works (Clarkson, 2002; Nuttall, 2004) indicating that most of Hg in urine is present as Hg(II). Our results are in agreement with those previous studies as more than 90% of the Hg found in urine is in the form of Hg(II) (Table S.4). This value is also in agreement with previous data of Hg species concentrations in urine (Nuttall, 2004). The median concentration of Hg(II) in urine was 1.3 ng Hg g^{-1} and the highest concentration was 5.8 ng Hg g^{-1} for a non-exposed individual (Table 2). In many cases, the levels of MeHg in urine were practically negligible and below the limit of quantification of the method. The median concentrations of Hg(II) in urine increase with the weekly frequency of fish consumption. However, there was no clear correlation between the levels of Hg(II) in urine with any of the other information showed in Table S.3.

3.2.3 Hg species concentration in hair

Similar MeHg levels in hair were found for both groups as the average concentrations for exposed and control individuals were 1.5 and 1.6 µg Hg g^{-1}, respectively. The levels of Hg(II) were slightly different between both groups (average concentrations of 0.3 and 0.1 µg g^{-1} for exposed and control

individuals, respectively). The obtained Hg(II) percentages were below 20% in most samples (except for individual 6, which was 53.3%) and consequently the MeHg percentages were above 80%. These results are in agreement with previous publications (Berglund et al., 2005; George et al., 2010). The general assumption that THg levels in hair are equivalent to MeHg concentrations is not valid for our set of samples. As expected, the median concentrations for MeHg of all individuals increased with fish consumption (Table 2) from 0.5 μg g^{-1} for non-fish consumers to 2.0 μg g^{-1} for individuals consuming fish 3 or more times per week. The concentration of hair MeHg obtained for the person who eats fish every day was also 2.0 μg g^{-1}. These levels can be influenced by the type of fish consumed but this factor could not be evaluated in our study. The sum of the MeHg and Hg(II) values in hair obtained in our work, are in agreement with the 95^{th} percentile (4.4 μg g^{-1}) of THg obtained for Spanish women reported in the European human biomonitoring study DEMOCOPHES (European Commission, 2012).

3.3 Additional factors influencing Hg species concentrations in the samples

Table 2 shows that MeHg levels in blood and hair vary depending on the consumption of tobacco. Smokers have a lower MeHg concentration in blood (median of 4.3 ng Hg g^{-1}) than ex-smokers (median of 10.4 ng Hg g^{-1}) and non-smokers (median 9.6 ng Hg g^{-1}). The same observation can be applied to hair samples. However, ex-smokers have a higher blood Hg(II) concentration (median of 2.9 ng Hg g^{-1}) than smokers and non-smokers (median of 0.9 and 0.7 ng Hg g^{-1} respectively). Different studies reporting lower concentrations of MeHg in smokers than in non-smokers (Jain, 2017; Lye et al., 2013). Jain attributed this observation to a possible interaction of some tobacco constituents with blood, which could favour the excretion or demethylation of MeHg. In addition, it has been reported that the consumption of alcohol may decrease Hg(II) levels in blood due to an inhibition of the activity of catalase by ethanol (Çoban et al., 2008). In our study, the participants

were not asked about alcohol consumption, but it could be a source of variability in Hg species concentrations.

Since we have observed a different proportion of Hg species in blood and urine in the oldest person of the study, an influence of age on the Hg species concentration cannot be ruled out. Other studies reported an increase of the MeHg concentration in blood with age (Mortensen et al., 2014; Sirot et al., 2008). However, we checked the correlation between the concentrations of the different mercury species with age but we did not find any significant correlation.

Individuals 5, 7, 26 and 27 reported to have dental amalgams with Hg. These amalgams have a composition of 50% metallic elemental Hg and other metals (Clarkson, 2002). Therefore, Hg^0 may be released from the amalgam and transformed into Hg (II) in blood (Halbach and Clarkson, 1978). We did not find a correlation between Hg(II) in blood and urine with the number of amalgams, but median Hg(II) concentration in urine of people reporting dental amalgams is higher than the median of people without dental fillings (1.7 versus 0.9 ng g^{-1}). Individual 27 from the control group informed that her dental fillings were manipulated a few days before sample collection and her Hg(II) concentration in urine was the highest from both groups (5.8 ng g^{-1}). Several works have studied the decrease of Hg(II) concentration over time after removing a dental amalgam (Björkman et al., 1997; Halbach et al., 2008).

3.4 Correlation between MeHg in blood with MeHg in hair

The MeHg hair-to-blood ratio was established as 250 by the Joint FAO / WHO Expert Committee on Food Additives (JECFA) in 2004, based on the average value obtained from different studies (JECFA-Joint FAO/WHO Expert Committee on food Additives, 2004). This value was calculated to facilitate the estimation of MeHg concentration in blood from the measured MeHg concentration in hair, which is a less invasive biomonitor. In our study, we have evaluated the hair-to-blood ratio for MeHg in both groups of samples. Figure 1 shows the correlation obtained for both groups. The

correlation coefficient obtained for the control group was r^2 = 0.8130 and that for the exposed individuals was r^2 = 0.8163. There is a clear difference in the slope of the linear regression between the two groups. In the case of the control group, a slope of 245 ± 42 equivalent to the reference value established by JECFA was obtained. However, the slope obtained for the group of exposed individuals was significantly lower (135 ± 17). The MeHg hair-to-blood ratios obtained for each individual is given in Figure S.1. As can be observed there are eight intoxicated workers with a MeHg hair-to-blood ratio under 200, indicating that the level of MeHg in blood is significantly higher than their expected level in hair in comparison with the control group and the JECFA reference value.

There are some publications reporting Hg hair-to-blood ratios (Berglund et al., 2005; Budtz-Jørgensen et al., 2004; Liberda et al., 2014; Yaginuma-Sakurai et al., 2012) between 200-370, but it should be mentioned that not all the studies were based on MeHg concentrations. Liberda and co-workers (2014) observed hair-to-blood ratios closer to 250 for fish consumers. This observation was also reported by Budtz-Jørgensen and co-workers (2004) for Faroese Children above 14 years old with a high fish consumption and a hair-to-blood ratio closed to 250 (median of 264). These results are in agreement with our data for the control group as their main Hg exposure is supposed to be fish consumption. According to this, when fish consumption is the main MeHg exposure, the ingested MeHg is excreted in hair resulting in a concentration ca. 250 times higher in hair than in blood.

It is interesting to note that individual 27 from the control group presented the highest MeHg hair-to-blood ratio in this study. As commented above, her dental amalgams were manipulated days before the collection of samples. This is consistent with other studies showing increased MeHg concentrations in hair (Sakamoto et al., 2007) and blood (Aitio et al., 1983) due to a recent exposition to Hg^0_v. Thus, a lower hair-to-blood ratio could indicate that part of MeHg present in blood is not coming directly from fish consumption but from other sources. The biomethylation of

Hg(II) deposits in different tissues or the remobilization of MeHg stored in tissues may be an explanation for these results for the intoxicated workers. Human in vivo methylation has never been proved to be responsible of a significant increase of MeHg levels in human biomonitors (Rodríguez Martín-Doimeadios et al., 2017), but it is known that some bacterial activity in mouth, gut or intestinal tract could be able to methylate Hg in a low extent (Leistevuo et al., 2001; Rowland et al., 1977; Rowland, 1995). The high MeHg levels in people exposed to high doses of Hg^0_v remains unclear.

3.5 Correlation of MeHg levels in hair between exposed and non-exposed relatives

Figure 2 shows the correlation between MeHg concentration in hair of the exposed individuals with MeHg concentration in hair of their relatives. A correlation coefficient of $r^2 = 0.9355$ was obtained, excluding an outlier corresponding to an exposed individual that lives in a different location than his relative (individuals 7 and 22). When comparing MeHg concentrations in blood (Figure S.2) no correlation was found between exposed and control individuals. The correlation shown in Figure 2 demonstrates the importance of lifestyle-related factors in the accumulation of MeHg in hair. The diet is the predominant source of MeHg incorporation into the body and therefore the main factor regulating MeHg accumulation in hair. The slope of the correlation (0.77 ± 0.08) indicates that non-exposed relatives have a higher concentration of MeHg in hair than exposed individuals probably due to their higher fish consumption levels (Table S.3). It is worth noting that MeHg concentrations in hair of three individuals having the lowest hair-to-blood ratios (individuals 1, 5, 8), correlate very well with their relatives.

3.6 Correlation between MeHg in hair and blood with Hg (II) in urine

311 Demethylation of MeHg in the organism has been widely reported (Dock et al., 1994; Suda and
312 Hirayama, 1992) although the nature of this process remains unknown. Measuring the isotopic
313 signature of Hg in blood and hair, Sherman et al. (2013) demonstrated that 70% of Hg(II) in urine
314 comes from MeHg demethylation in people with less than ten dental amalgams consuming fish
315 regularly.

316 Measuring Hg species concentrations, we were able to study the correlation of MeHg concentration
317 in hair with Hg (II) concentration in urine. Figure 3 show such correlation between the exposed
318 individuals and the control group. No significant difference was obtained between the slope
319 obtained for the exposed individuals (883 ± 150) and that obtained for the control group (731 ±
320 183). The correlation coefficients (r^2) were 0.7119 and 0.6950 for exposed and the control group,
321 respectively. It must be taken into account that two outliers were found in these correlation,
322 corresponding to the individuals with the highest Hg(II) levels in urine (individuals 9 and 27). As
323 commented before, individual 27 suffered a possible recent exposure to Hg^0_v derived from her
324 dental amalgams, which, according to Sherman et al. (2013), could significantly modify the
325 correlation of Hg (II) in urine with MeHg demethylation. Also, Halbach et al. (2008) observed an
326 increment in blood MeHg due to the removal of Hg dental fillings. However, in the case of
327 individual 9, we could not find any reason explaining the different behaviour in comparison with
328 the other individuals.

329 The correlation of MeHg in blood with Hg (II) in urine is not as clear as that for MeHg in hair with
330 Hg (II) in urine. Figure S.3 of the Supplementary material shows that we could find a correlation
331 (excluding individual 27) for the control group obtaining a correlation coefficient of r^2=0.6646 and
332 a slope of 3.03 ± 0.81. Some of the exposed individuals fit well in the correlation of non-exposed
333 individuals, but those presenting the lowest MeHg hair-to-blood ratios (individuals 5, 7, 8, 11, 12,
334 14, 16) are out of this correlation. The ratios of blood MeHg-to-urine Hg (II) for these individuals
335 are much higher than those obtained for the control group. This means that these individuals present

a higher concentration of MeHg in blood than that expected from their Hg(II) concentration in urine. These results are in agreement with the possible remobilization of Hg stored in tissues to increase the concentration of MeHg in blood of the exposed group.

3.7 Summary of the correlation between Hg species concentrations

If we summarized all the correlations between species, we can conclude first that the excretion of Hg(II) in urine is correlated with MeHg concentration in hair (Figure 3), both for the control group and the exposed individuals. This evidence was first described by Sherman and co-workers (2013) by means of stable isotope analysis of Hg in hair and urine. Also, when comparing the concentrations of MeHg in hair we obtained a correlation between the exposed individuals and their relatives with a slope of 0.77 and a regression coefficient of 0.9355 (Figure 2). However, we found a significant difference between the exposed individuals and the control group, when comparing MeHg concentration in blood (Figure S.2). In contrast to the control group, most of the exposed individuals showed MeHg levels in blood higher than those expected according to their Hg (II) concentration in urine (Figure S.3) and their MeHg concentrations in hair (Figure 1).

All these observations suggest that for exposed individuals there is a different correlation of MeHg in blood with other factors. However, MeHg levels in hair for the exposed individuals follow the same correlations than the control group. The strong correlation between Hg in contaminated fish and Hg in human hair applying stable isotope analysis has been previously reported (Li et al., 2014). Our results agree with the assumption that MeHg in hair seems to come mainly from the diet, both for the exposed individuals and for the control group.

The lower MeHg hair-to-blood ratios for the exposed individuals indicate that part of the MeHg in blood should come from another source. There are eight intoxicated workers with MeHg hair-to blood lower than 200 and MeHg blood-to-Hg (II) urine ratios higher than the average ratio for the

control group. We postulate here that this excess of MeHg in blood may come from Hg stores in different tissues, such as kidney or liver, and that those internal stores are not reflected in an increased MeHg in hair. Indeed, the high correlation of MeHg concentrations in hair between the exposed workers and their relatives suggests that the MeHg ingested by the diet follows the same pathway for both groups. We investigated the possibility of a high uptake of MeHg through diet in the days before sample collection that could be detected in blood but not detected in hair but it was denied by the exposed individuals. Most of the exposed individuals have moderate fish consumption as they were advised after the intoxication to reduce and even avoid fish consumption altogether by medical prescription. Four of them do not consume any fish and the rest do it moderately. For example, among the non-fish consumers, individual 14 showed the highest MeHg levels in hair and blood, and a MeHg hair-to-blood ratio of 158.

These results are only consistent with the hypothesis that there are two different MeHg sources in blood. One would correspond to the MeHg ingested through the diet that follows a classical deposition process in hair and a further demethylation process to be excreted through urine as Hg(II). The other source must be the result of a metabolic pathway occurring when a high concentration of Hg is stored in the organism. This could explain the high levels of MeHg in people with a moderate consumption of fish or even non-fish consumers. There are several studies of people highly exposed to Hg^0_v showing increased organic Hg levels in blood. One hypothesis for this evidence described two binding sites for Hg in the kidney with different affinity (Clarkson and Magos, 1966), which in the case of an excess of Hg^0_v would cause a redistribution of MeHg. Ishihara et al. (1977) used this hypothesis to explain the increase of the organic Hg levels in blood but not in the hair of women exposed to Hg^0_v. Suzuki and co-workers (1976) described a weaker interaction of MeHg with tissues than that of Hg (II). In addition, the presence of Hg (II) induces renal metallothionein biosynthesis and gluthathione reduction as mechanisms to reduce Hg toxicity (Cherian and Clarkson, 1976; Halbach et al., 2008). Halbach and co-workers (2008) explained this

procedure as an intra-extra cellular exchange of Hg species mediated by GSH (Gluthatione) with a preferential affinity by Hg (II) than MeHg. After Hg(II) detoxification, MeHg could occupy the positions of Hg (II), increasing its concentration in erythrocytes. The different intra and extra cellular mechanisms together with the equilibrium established by the different compartments may be responsible of a redistribution of Hg species in blood and these procedures could be influenced by a high exposition to Hg^0_v.

3.8 Comparison with other studies on Hg in different populations.

Average Hg species concentrations obtained in this study do not differ significantly between the exposed individuals and the control group. There are established limits such as NOAEL (No-Observed-Adverse-Effect-Level), LOAEL (Lowest-Observed-Adverse-Effect-Level), HBM levels (Human Biomonitoring), BAT (Biologischer Arbeitsstoff-Toleranzwert) and, PWTI (Provisional Tolerable Weekly Intake) that regulate Hg intake by fish consumption and occupational exposure to Hg^0_v. However, large differences between Hg levels of different populations have been reported. We have compared the Hg species concentrations obtained in our study with THg levels in blood previously reported in the literature.

Although speciation methodologies for Hg are scarce in human biomonitoring studies, some of the studies reported MeHg concentrations in blood. Figure 4 shows the comparison of THg and MeHg levels in blood obtained from different works. As can be observed, most of the studies show an average concentration of THg or MeHg in blood below 5 ng (of Hg) g^{-1}. This concentration corresponds to the HBM-I level, which indicates the Hg body burden that does not present any risk to health established by the German Human Biomonitoring (HBM) commission (Apel et al., 2017; World Health Organization & UNEP, 2008). The studies reporting low THg levels in blood belong to different European countries (Berglund et al., 2005; Björnberg et al., 2005; Gibb et al., 2011; Gundacker et al., 2010; Lindberg et al., 2004; Puklová et al., 2010; Reis et al., 2007;Rignell-

410 Hydbom et al., 2007; Vahter et al., 2000) and large population studies of Canada and U.S. (CDC-
411 US, 2017; Lye et al., 2013; Mortensen et al., 2014). These studies report that the higher values of
412 THg or MeHg in blood correspond to fish or seafood consumers such as the French coastal
413 population, fishermen and relatives in Finland (Airaksinen et al., 2010; Sirot et al., 2008) or Faroese
414 children in which the diet is highly influenced by pilot whale meat (Budtz-Jørgensen et al., 2004).

415 Only three of the studies present concentrations in blood above 25 ng (of Hg) g^{-1}, which is the THg
416 threshold value defined by BAT ("Biologischer Arbeitsstoff-Toleranz-Wert") as the maximum
417 allowable concentration of a substance or its metabolites in body. The studies reporting higher
418 concentrations explain Hg exposure by fish consumption (Carta et al., 2003; Choi et al., 2009) or by
419 living or working in areas of artisanal gold mining activities (Dolbec et al., 2000). Another study
420 related to people who follow a high fish consumption behaviour in Sweden (Björnberg et al., 2005)
421 shows that the THg levels are much lower than those found in similar studies conducted in Spain.
422 The same observation was also reflected in the DEMOCOPHES (European Commission, 2012)
423 study in hair between Swedish and Spanish women.

424 The average concentration of the individuals in our study is comparable to the concentrations
425 reported for people of the same region, with and without occupational exposure. In addition, there
426 are three studies with concentrations of THg in blood in the same range (Baeuml et al., 2011; Jo et
427 al., 2015; Yaginuma-Sakurai et al., 2012). This data is consistent with our data between the
428 intoxicated individuals and the control group. Surprisingly, people in this region have blood THg
429 values comparable to individuals from contaminated regions due to artisanal gold mining (Baeuml
430 et al., 2011). THg levels in blood of shellfish consumers of the French coast (Sirot et al., 2008) are
431 also closed to the average value of THg in blood in our study. In the light of these studies, we want
432 to highlight the importance of speciation analysis, which could, for example, differentiate between
433 populations with a high consumption of contaminated fish, or with a high exposure to Hg^0_v. Studies

reporting concentrations of the different Hg species would provide more information about contamination sources than those based only on THg concentrations.

4. CONCLUSIONS

This is the first study using a triple spike IDMS methodology reporting the levels of different mercury compounds in the three most commonly used human biomonitors (blood, urine and hair). Only one previous study (Akagi *et al*. 1995) also analyzed blood, urine and hair of the same individuals but determined only total mercury and methylmercury. Also, they did not employ any method to correct for species transformation. So, we present the first case study employing the most advanced analytical procedure for mercury to date.

The Hg species concentrations found in this study are similar to those obtained in previous studies carried out in the same region and in different regions with people highly exposed to MeHg intake by fish consumption. The concentrations obtained in the exposed individuals were not significantly different from those obtained in the control group, except for MeHg in blood. High MeHg levels obtained in some intoxicated individuals were surprising as they avoid fish consumption since the accident. A different correlation between MeHg in hair and MeHg in blood was obtained for these individuals compared to the control group who showed a hair-to-blood ratio (245) consistent with the reported value for people exposed to Hg via fish consumption (250).

We found a correlation of MeHg in hair between the exposed individuals and the control group reflecting that MeHg excretion in hair follows the same pathways in both groups and depends on lifestyle factors such as fish consumption. We obtained also a correlation of MeHg in hair with Hg (II) in urine. The individuals showing lower MeHg hair-to-blood ratios showed higher MeHg blood-to-urine Hg(II) ratios than the control group. These observations suggest that some of the workers exposed to Hg_v^0 show an increased MeHg level in blood after three years of the accident and that they do not follow the same MeHg excretion mechanisms (deposition in hair and demethylation and excretion through urine). We find very difficult to explain why a person who does not eat fish

present a MeHg concentration of 21.6 ng (of Hg) g^{-1} in his blood. At this point we can only hypothesize on MeHg remobilization or biomethylation processes to explain those abnormal MeHg levels in blood. Hg species have not been studied in individuals exposed to toxic levels of Hg_v^0 thus far so the available information on the Hg species distribution in humans is very limited.

5. ACKNOWLEDGEMENTS

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Figures and tables

Figure 1. MeHg concentration in hair samples (ng Hg g^{-1}) versus MeHg concentration in blood samples (ng Hg g^{-1}) of the exposed individuals and the control group.

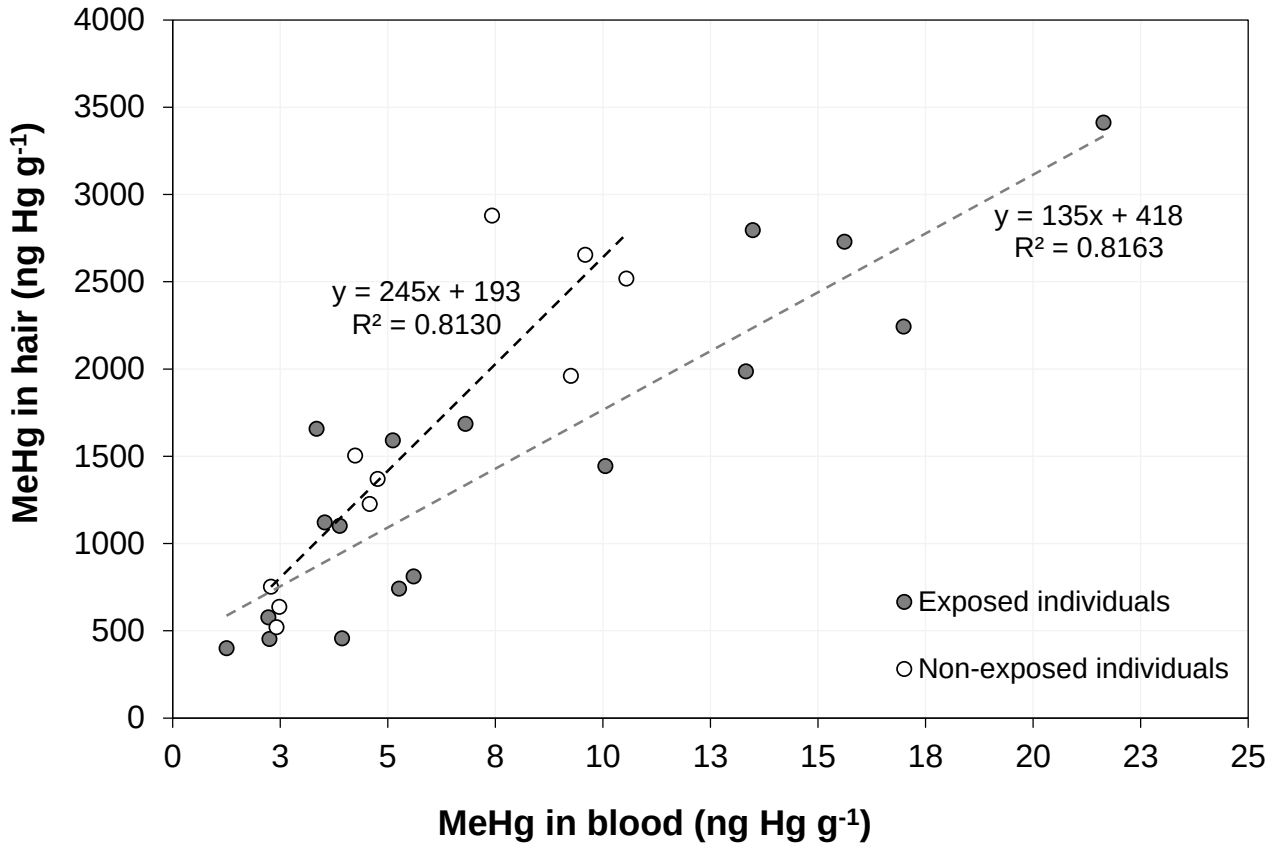
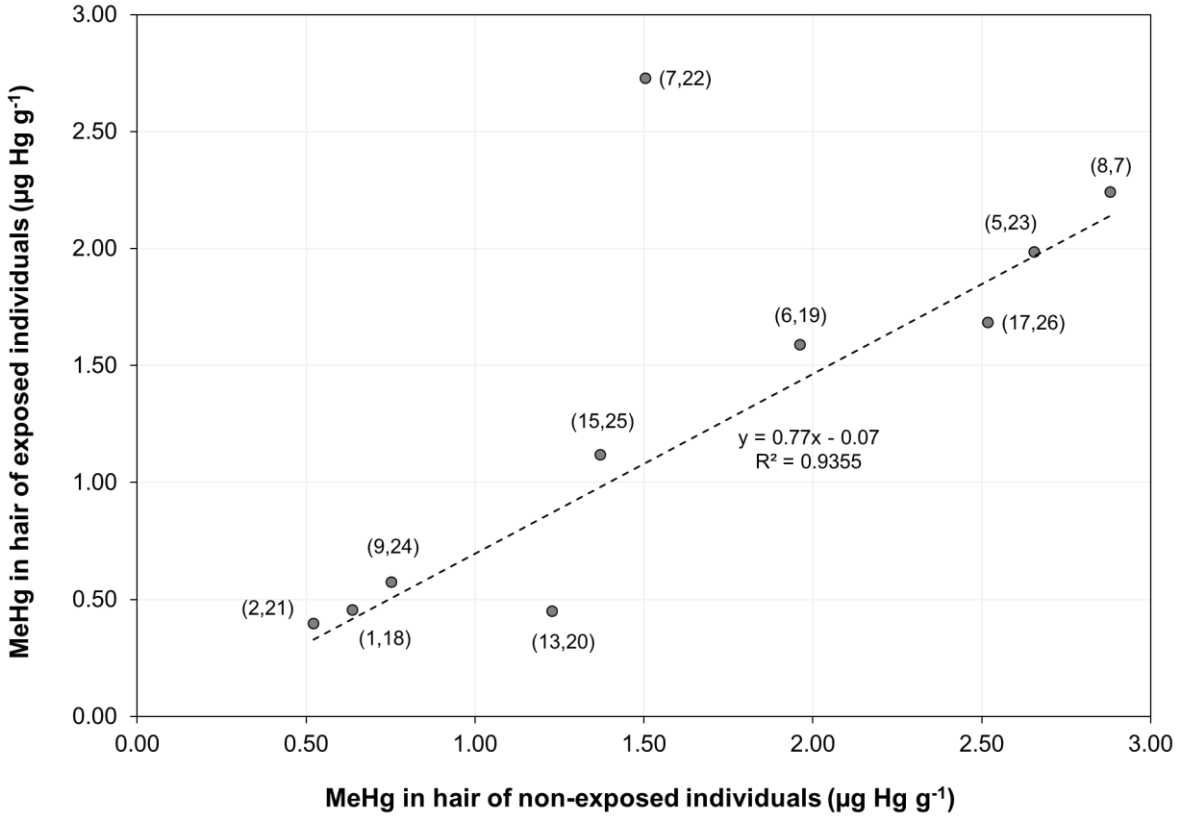


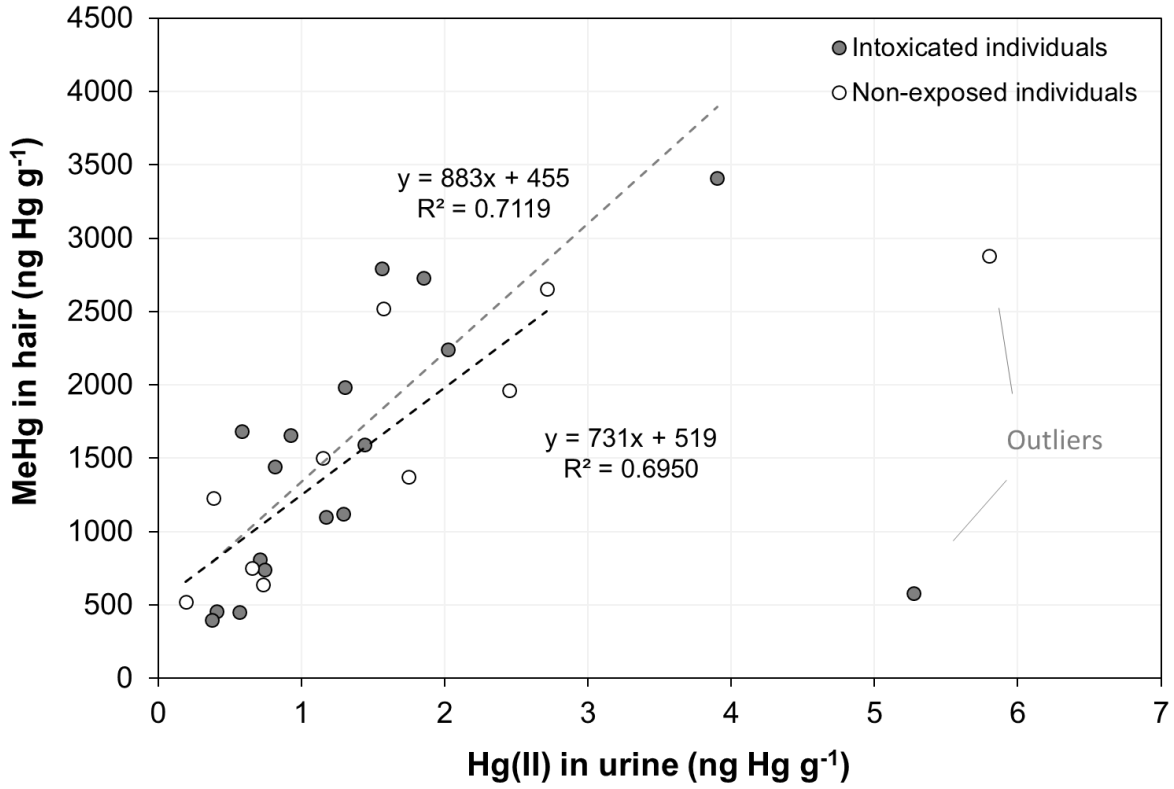
Figure 2. MeHg concentrations in hair samples of exposed individuals (µg Hg g^{-1}) versus MeHg concentration in hair samples of their corresponding relatives (µg Hg g^{-1}). The numbers in brackets indicate the identification of the exposed individual and his relative.



714 **Figure 3.** MeHg concentrations in hair samples (ng Hg g^{-1}) versus Hg (II) concentrations in urine

715 samples (ng Hg g^{-1}) of exposed and non-exposed individuals.

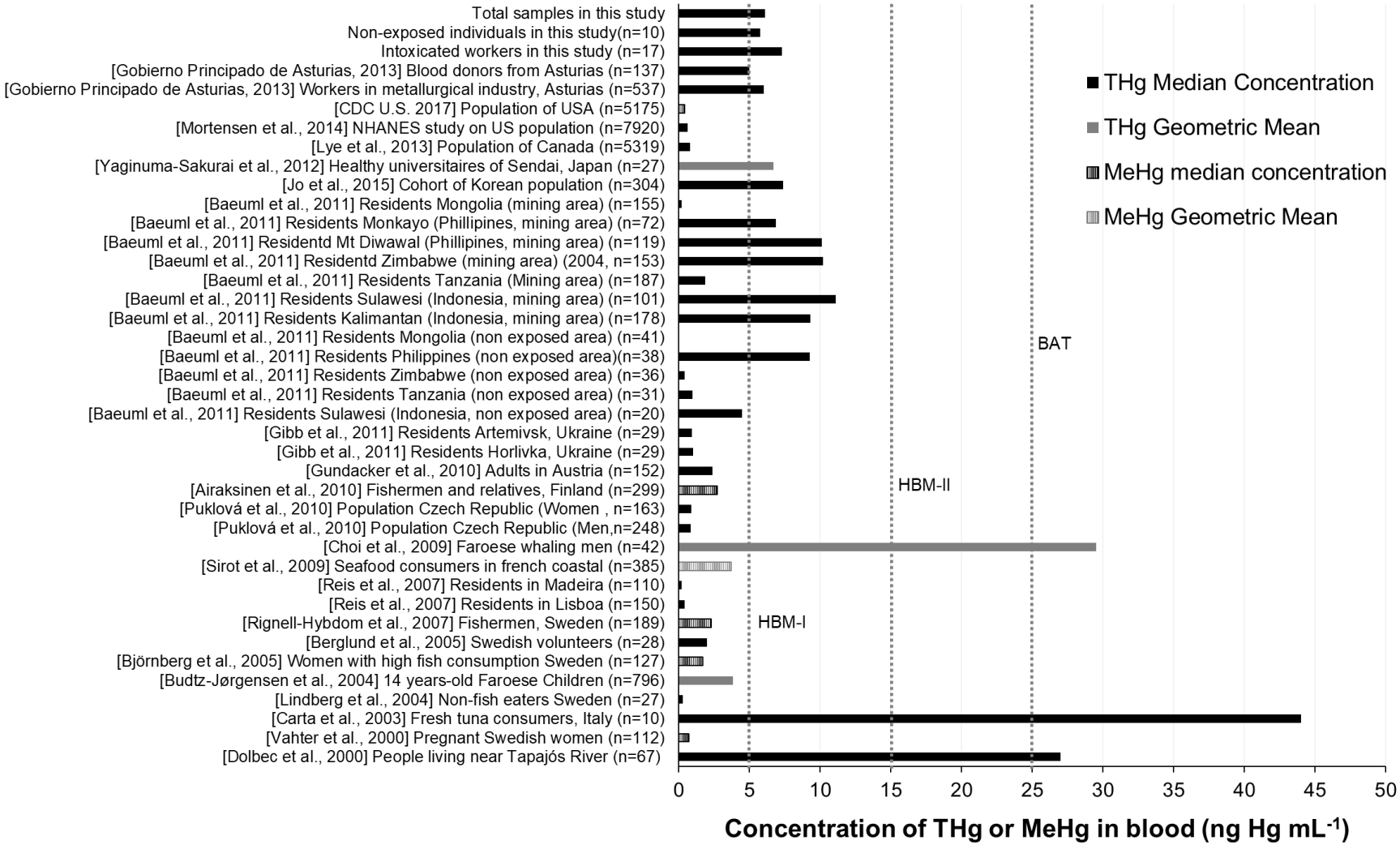
716



717

718

719 **Figure 4.** Comparison of THg and MeHg concentrations in blood obtained in this work with those obtained in other studies (ng mL^{-1}). The
720 vertical dot-lines represent the threshold values (HBM-I, HBM-II, BAT) for risk assessment on mercury concentrations.



721

722 **Table 1**. Individual concentrations of Hg(II) and MeHg obtained in urine, blood and hair of the
723 exposed individuals and the control group.

		Urine (ng Hg g^{-1})		Blood (ng Hg g^{-1})		Hair (µg Hg g^{-1})	
	Sample	**Hg(II)**	**MeHg**	**Hg(II)**	**MeHg**	**Hg(II)**	**MeHg**
Exposed Individuals	1	0.41	0.01	0.67	3.94	0.04	0.46
	2	0.37	0.00	0.29	1.25	0.05	0.40
	3	1.17	0.01	0.67	3.88	0.07	1.10
	4	1.56	0.01	0.71	13.48	0.31	2.80
	5	1.30	0.05	4.12	13.33	0.13	1.99
	6	1.44	0.04	0.97	5.11	1.84	1.59
	7	1.85	0.08	0.35	15.62	0.27	2.73
	8	2.02	0.05	0.31	16.99	0.23	2.24
	9	5.28	0.20	1.61	2.22	0.13	0.58
	10	0.92	0.06	1.82	3.34	0.10	1.66
	11	0.71	0.05	1.80	5.60	0.06	0.81
	12	0.74	0.04	3.61	5.26	0.08	0.74
	13	0.57	0.04	0.47	2.25	0.10	0.45
	14	3.90	0.11	0.84	21.64	0.24	3.41
	15	1.29	0.05	1.17	3.53	0.09	1.12
	16	0.82	0.06	0.54	10.06	0.12	1.44
	17	0.58	0.06	0.49	6.80	0.29	1.69
Control Group	18	0.73	0.03	0.94	2.48	0.03	0.64
	19	2.45	0.06	0.82	9.26	0.40	1.96
	20	0.39	0.02	1.51	4.58	0.09	1.23
	21	0.19	0.02	0.51	2.41	0.05	0.52
	22	1.15	0.06	0.74	4.24	0.09	1.50
	23	2.71	0.09	0.81	9.59	0.16	2.65
	24	0.66	0.08	2.61	2.28	0.11	0.75
	25	1.75	0.06	0.67	4.76	0.09	1.37
	26	1.57	0.06	4.26	10.55	0.15	2.52
	27	5.80	0.11	2.20	7.42	0.11	2.88

Table 2. Descriptive statistics (geometric means (GM), medians (50th percentile), 95th percentiles and minimum (Min) and maximum (Max) values) for MeHg and Hg(II) concentrations in blood, hair and urine for all the individuals divided into classified groups.

		MeHg in blood (ng (of Hg) g^{-1})			Hg(II) in blood (ng (of Hg) g^{-1})			MeHg in hair (µg (of Hg) g^{-1})			Hg(II) in hair (µg (of Hg) g^{-1})			MeHg in urine (ng (of Hg) g^{-1})			Hg(II) in urine (ng (of Hg) g^{-1})		
	N	GM	50th -95th Percentile	Min-Max	GM	50th -95th Percentile	Min-Max	GM	50th -95th Percentile	Min-Max	GM	50th -95th Percentile	Min-Max	GM	50th -95th Percentile	Min-Max	GM	50th -95th Percentile	Min-Max
Total of individuals	27	7.1 ± 5.4	5.1 - 16.7	1.3 - 21.6	1.3 ± 1.2	0.8 - 4.0	0.3 - 4.3	1.5 ± 0.9	1.4 - 2.9	0.4 - 3.4	0.2 ± 0.4	0.1 - 0.4	0.0 - 1.8	0.1 ± 0.0	0.0 - 0.1	0.0 - 0.2	1.6 ± 1.5	1.3 - 5.0	0.2 - 5.8
Men (exposed workers)	17	8.1 ± 6.4	5.3 - 18.4	1.3 -21.6	1.2 ± 1.2	0.7 - 3.8	0.3 - 4.1	1.5 ± 1.0	1.1 - 3.0	0.4 - 3.4	0.3 ± 0.4	0.1 - 0.8	0.0 - 1.8	0.1 ± 0.0	0.0 - 0.1	0.0 - 0.2	1.5 ± 1.4	1.3 - 4.3	0.4 - 5.3
Women (control group)	10	5.8 ± 3.2	4.7 - 10.1	2.3 - 10.5	1.5 ± 1.2	0.9 - 3.5	0.5 - 4.3	1.6 ± 0.9	1.4 - 2.8	0.5 - 2.9	0.1 ± 0.1	0.1 - 0.3	0.0 - 0.4	0.1 ± 0.0	0.1 - 0.1	0.0 - 0.1	1.7 ± 1.7	1.4 - 4.4	0.2 - 5.8
Smokers	12	4.3 ± 2.2	4.3 - 7.9	1.3 - 9.3	1.0 ± 0.5	0.9 - 1.7	0.3 - 1.8	1.0 ± 0.5	1.0 - 1.8	0.4 - 2.0	0.3 ± 0.5	0.1 - 1.0	0.0 - 1.8	0.1 ± 0.1	0.0 - 0.1	0.0 - 0.2	1.3 ± 1.4	0.7 - 3.7	0.4 - 5.3
Ex -Smokers	4	10.8 ± 5.4	10.4 - 16.4	5.3 - 17.0	2.6 ± 1.7	2.9 - 4.0	0.3 - 4.1	2.0 ± 0.9	2.1 - 2.8	0.7 - 2.9	0.1 ± 0.1	0.1 - 0.2	0.1 - 0.2	0.1 ± 0.0	0.0 - 0.1	0.0 - 0.1	2.5 ± 2.3	1.7 - 5.2	0.7 - 5.8
Non- Smokers	9	9.3 ± 6.7	9.6 - 19.2	2.3 - 21.6	1.3 ± 1.3	0.7 - 3.6	0.3 - 4.3	2.0 ± 1.0	2.5 - 3.2	0.5 - 3.4	0.2 ± 0.1	0.1 - 0.3	0.0 - 0.3	0.1 ± 0.0	0.1 - 0.1	0.0 - 0.1	1.6 ± 1.1	1.6 - 3.4	0.2 - 3.9
Fish consumption																			
No consumption	5	6.3 ± 8.6	2.5 - 18.1	1.3 - 21.6	0.6 ± 0.3	0.7 - 0.9	0.3 - 0.9	1.1 ± 1.3	0.5 - 2.9	0.4 - 3.4	0.1 ± 0.1	0.0 - 0.2	0.0 - 0.0	0.0 ± 0.1	0.0 - 0.1	0.0 - 0.1	1.2 ± 1.5	0.6 - 3.3	0.4 - 3.9
1 time per week	4	4.0 ± 1.3	4.2 - 5.2	2.3 - 5.3	2.1 ± 1.3	2.1 - 3.5	0.7 - 3.6	1.0 ± 0.2	0.9 - 1.2	0.7 - 1.2	0.1 ± 0.0	0.1 - 0.1	0.1 - 0.1	0.0 ± 0.0	0.0 - 0.1	0.0 - 0.1	0.7 ± 0.3	0.7 - 1.1	0.4 - 1.2
2 times per week	9	9.1 ± 5.8	6.8 – 16.4	2.2 – 17.0	1.2 ± 1.2	0.7 – 3.2	0.3 – 4.1	1.7 ± 0.9	1.7 – 2.8	0.5 – 2.8	0.4 ± 0.6	0.2 – 1.2	0.0 – 1.8	0.1 ± 0.1	0.0 – 0.2	0.0 – 0.2	1.7 ± 1.5	1.4 – 4.0	0.2 – 5.3
3-4 times per week	6	6.7 ± 2.9	6.1 – 10.3	3.5 – 10.5	1.6 ± 1.4	1.0 – 3.7	0.7 – 4.3	2.0 ± 0.8	2.0 – 2.8	1.1 – 2.9	0.1 ± 0.0	0.1 – 0.2	0.1 – 0.2	0.1 ± 0.0	0.1 – 0.1	0.0 – 0.1	2.4 ± 1.8	1.7 – 5.0	1.2 – 5.8
Everyday	1	9.3	9.3	9.3	0.8	0.8	0.8	2.0	2.0	2.0	0.4	0.4	0.4	0.1	0.1	0.1	2.4	2.4	2.4
Poultry consumption																			
No consumption	4	8.5 ± 5.3	7.2 - 14.7	3.9 - 15.6	0.7 ± 0.3	0.7 - 0.9	0.3 - 1.0	1.7 ± 0.9	1.8 - 2.6	0.5 - 2.7	0.6 ± 0.8	0.3 - 1.6	0.0 - 1.8	0.0 ± 0.0	0.0 - 0.0	0.0 - 0.0	1.5 ± 0.9	1.6 - 2.4	0.4 - 2.4
1 time per week	3	3.7 ± 1.3	4.2 - 4.5	2.2 - 4.6	0.9 ± 0.5	0.7 - 1.4	0.5 - 1.5	1.1 ± 0.5	1.2 - 1.5	0.5 - 1.5	0.1 ± 0.0	0.1 - 0.1	0.1 - 0.1	0.0 ± 0.0	0.0 - 0.1	0.0 - 0.1	0.7 ± 0.4	0.6 - 1.1	0.4 - 1.2
2 times per week	17	7.7 ± 5.8	5.6 - 17.9	1.3 - 21.6	1.6 ± 1.3	0.8 - 4.1	0.3 - 4.3	1.6 ± 1.0	1.4 - 3.0	0.4 - 3.4	0.1 ± 0.1	0.1 - 0.3	0.0 - 0.3	0.1 ± 0.0	0.1 - 0.1	0.0 - 0.2	1.9 ± 1.7	1.3 - 5.4	0.2 - 5.8
> 2 times per week	1	2.5	2.5	2.5	0.9	0.9	0.9	0.6	0.6	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.7	0.7
Beef consumption																			
1 time per week	8	7.7 ± 5.3	6.5 - 15.7	2.2 - 17.0	1.8 ± 1.2	1.7 - 3.6	0.3 - 4.1	1.7 ± 0.9	1.7 - 2.8	0.6 - 2.9	0.1 ± 0.0	0.1 - 0.2	0.1 - 0.2	0.1 ± 0.1	0.1 - 0.2	0.0 - 0.2	2.5 ± 2.0	1.7 - 5.6	0.7 - 5.8
2 times per week	17	6.9 ± 5.6	4.8 - 16.8	1.3 - 21.6	1.1 ± 1.1	0.7 - 3.7	0.3 - 4.3	1.5 ± 0.9	1.2 - 2.9	0.4 - 3.4	0.2 ± 0.4	0.1 - 0.7	0.0 - 1.8	0.0 ± 0.0	0.0 - 0.1	0.0 - 0.1	1.2 ± 0.9	1.2 - 2.7	0.2 - 3.9
Pork consumption																			
No consumption	1	9.3	9.3	9.3	0.8	0.8	0.8	2.0	2.0	2.0	0.4	0.4	0.4	0.1	0.1	0.1	2.4	2.4	2.4
1 time per week	6	5.5 ± 2.9	4.6 - 9.6	2.5 - 10.5	1.8 ± 1.7	0.8 - 4.1	0.5 - 4.3	1.2 ± 0.8	0.9 - 2.3	0.5 - 2.5	0.1 ± 0.1	0.1 - 0.3	0.0 - 0.3	0.0 ± 0.0	0.0 - 0.1	0.0 - 0.1	0.9 ± 0.4	0.7 - 1.5	0.4 - 1.6
2 times per week	17	7.8 ± 6.2	5.1 - 17.9	1.3 - 21.6	1.2 ± 1.0	0.8 - 2.9	0.3 - 4.1	1.6 ± 1.0	1.5 - 3.0	0.4 - 3.4	0.2 ± 0.4	0.1 - 0.6	0.0 - 1.8	0.1 ± 0.0	0.1 - 0.1	0.0 - 0.2	1.9 ± 1.7	1.4 - 5.4	0.2 - 5.8
> 2 times per week	1	3.5	3.5	3.5	1.2	1.2	1.2	1.1	1.1	1.1	0.1	0.1	0.1	0.0	0.0	0.0	1.3	1.3	1.3
Age																			
30-39 yr	4	4.5 ± 0.9	4.5 - 5.5	3.5 - 5.6	1.1 ± 0.5	1.0 - 1.7	0.7 - 1.8	1.2 ± 0.3	1.2 - 1.5	0.8 - 1.5	0.1 ± 0.0	0.1 - 0.1	0.1 - 0.1	0.1 ± 0.0	0.1 - 0.1	0.0 - 0,1	1.2 ± 0.4	1.2 - 1.7	0.7 - 1.7
40-49 yr	11	9.2 ± 6.5	9.6 - 18.6	1.3 - 21.6	1.3 ± 1.5	0.7 - 4.2	0.3 - 4.3	1.9 ± 1.1	2.0 - 3.1	0.4 - 3.4	0.2 ± 0.1	0.1 - 0.3	0.0 - 0.2	0.1 ± 0.1	0.1 - 0.2	0.0 - 0.2	1.9 ± 1.6	1.6 - 4.6	0.2 - 5.3
50-59 yr	8	6.5 ± 4.9	4.8 - 14.3	2.2 - 17.0	1.0 ± 0.6	0.9 - 2.0	0.3 - 2.2	1.4 ± 0.9	1.4 - 2.7	0.5 - 2.9	0.4 ± 0.6	0.1 - 1.3	0.0 - 1.8	0.0 ± 0.0	0.0 - 0.1	0.0 - 0.1	1.7 ± 1.8	1.1 - 4.6	0.4 - 5.8
≥60 yr	2	3.8 ± 2.1	3.8 - 5.1	2.3 - 5.3	3.1 ± 0.7	3.1 - 3.6	2.6 - 3.6	0.7 ± 0.0	0.7 - 0.8	0.7 - 0.8	0.1 ± 0.0	0.1 - 0.1	0.1 - 0.1	0.1 ± 0.0	0.1 - 0.1	0.0 - 0.1	0.7 ± 0.1	0.7 - 0.7	0.7 - 0.7
Dental amalgams																			
Yes	4	11.7 ± 3.5	11.9 - 15.3	7.4 - 15.6	2.7 ± 1.8	3.2 - 4.2	0.3 - 4.3	2.5 ± 0.4	2.6 - 2.9	2.0 - 2.9	0.2 ± 0.1	0.1 - 0.3	0.1 - 0.3	0.1 ± 0.0	0.1 ± 0.1	0.0 - 0.1	2.6 ± 2.1	1.7 - 5.2	1.3 - 5.8
No	21	6.3 ± 5.1	4.6 - 16.6	1.3 - 21.6	1.1 ± 0.8	0.8 - 2.5	0.3 - 3.6	1.3 ± 0.9	1.1 - 2.8	0.4 - 3.4	0.2 ± 0.4	0.1 - 0.4	0.0 - 1.8	0.1 ± 0.0	0.0 - 0.1	0.0 - 0.2	1.4 ± 1.2	0.9 - 3.8	0.2 - 5.3