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EVALUATION OF GLUTATHIONE *S*-TRANSFERASE *GSTM1* AND *GSTT1* POLYMORPHISMS AND METHYLMERCURY METABOLISM IN AN EXPOSED AMAZON POPULATION

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Over the last decades, the presence of methylmercury (MeHg) in the Amazon region of Brazil and its adverse human health effects have given rise to much concern. The biotransformation of MeHg occurs mainly through glutathione (GSH) in the bile mediated by conjugation with glutathione *S*-transferases (GST). Epidemiological evidence has shown that genetic polymorphisms may affect the metabolism of MeHg. The aim of this study was to evaluate the association between GST polymorphisms, GSH, and Hg levels in blood (B-Hg) and in hair (H-Hg) of an Amazon population chronically exposed to the metal through fish consumption. Blood and hair samples were collected from 144 volunteers (71 men, 73 women). B-Hg and H-Hg levels were determined by inductively coupled plasma–mass spectrometry, and GSH levels were evaluated by a spectrophotometric method. *GSTM1* and *T1* genotyping evaluation were carried out by multiplex polymerase chain reaction (PCR). Mean levels of B-Hg and H-Hg were 37.7 ± 24.5 μg/L and 10.4 ± 7.4 μg/g, respectively; GSH concentrations ranged from 0.52 to 2.89 μ*M*/ml of total blood. Distributions for *GSTM1/T1*, *GSTM1/GSTT1*0*, *GSTM1*0/T1*, and *GSTM1*0/GSTT1*0* genotypes were 35.4, 22.2, 25.0, and 17.4%, respectively. *GSTT1* genotype carriers presented lower levels of B-Hg and H-Hg when compared to other genotypes carriers. In addition, *GSTM1*0/GSTT1*0* individuals presented higher Hg levels in blood and hair than subjects presenting any other genotypes. There appeared to be no evidence of an effect of polymorphisms on GSH levels. Therefore, our data suggest that GST polymorphisms may be associated with MeHg detoxification.

Mercury (Hg) is one of the most hazardous metals in the environment, and metal-mediated toxicity involves oxidative damage to macromolecules (Beyersmann and Hartwig 2008; Chuu et al. 2008; Mori et al. 2007). Several experimental and epidemiological studies demonstrated that exposure to Hg in its organic form, methylmercury (MeHg), which is found in fish and other seafood, is associated with neurotoxic effects (Counter et al. 2002; Dolbec et al. 2000), damage to the immune and renal system (Moszczynski et al. 1998; Rutowski et al. 1998), infertility (Boujbiha et al. 2009), cardiovascular diseases (Virtanen et al. 2007), and cancer (IARC 1993).

Fish is considered a healthy food because it is rich in proteins, poor in saturated fats, and protects against coronary diseases (Millen and Quatromoni 2001; Whelton et al. 2004). Populations that traditionally consume large

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amounts of fish generally present low rates of mortality from heart disease (Dewailly et al. 2001; 2002). On the other hand, fish consumption is also an important source of human exposure to a variety of bioaccumulative xenobiotics such as MeHg (Burger et al. 2003; Clarkson 2002; Johnsson et al. 2005), which may counteract the beneficial effects of fish consumption (Chan and Egeland 2004; Grotto et al. 2011; Stern 2005).

In the Amazon region, MeHg exposure through fish consumption has been the focus of much concern over the last two decades. In many parts of the Amazon region, a large number of exposure assessments of fish-eating populations have been conducted using blood and/or hair as biomarkers to evaluate communities environmentally exposed to the metal through the diet (Dórea et al. 2005; Gonçalves and Gonçalves, 2004; Lebel et al. 1997; Passos et al. 2003; Pinheiro et al. 2000). These findings have consistently shown elevated blood and/or hair Hg concentrations correlated to fish consumption. Moreover, dose-related deficits in neurobehavioral and neurophysiological functions have been found in both children and adults (Cordier et al. 2002; Dolbec et al. 2000; Grandjean et al. 1999; Lebel et al. 1996), and there is also evidence of cardiotoxicity associated with Hg exposure in some Amazon communities (Fillion et al. 2006).

Methylmercury elimination in humans is related to the glutathione (GSH) detoxification system in bile, mediated by conjugation with glutathione *S*-transferases (GST), which produces a stable GSH–metal conjugate in hepatic tissue and then, eliminated mainly via feces (Ballatori and Clarkson 1985). Several GSH-related enzymes are highly polymorphic, and epidemiological studies demonstrated that these polymorphisms may affect the metabolism of Hg (Goyer and Clarkson 2001; Gundacker et al. 2010). Moreover, other studies were carried out concerning the relationship between other polymorphisms and adverse health effects induced by Hg exposure (de Marco et al. 2011; Echeverria et al. 2010; Heyer et al. 2009). The GST genes, especially *GSTM1* (mu) and *T1* (theta), are important since they present a deletion that results in impaired catalytic activity associated with greater sensitivity to toxic compounds. The homozygous deletion *GSTM1 (M1*0)* and *GSTT1 (T1*0)* genotypes are generally associated with a high risk of cancer and development of other diseases (Ginsberg et al. 2009; Gundacker et al. 2007; 2010). However, there are few data with respect to the influence of GST polymorphisms on Hg detoxification in humans.

Since Hg elimination is related to GSH conjugation, the aims of the present study were to (1) determine the genotype frequency of the *GSTM1* and *GSTT1* polymorphisms and (2) examine the influence of *GSTM1*0* and *GSTT1*0* polymorphisms on blood-Hg (B-Hg), hair-Hg (H-Hg) and GSH levels of a group of riparian individuals exposed to MeHg via consumption of contaminated fish from the Tapajós River in the Amazon region of Brazil.

MATERIALS AND METHODS

Study Design and Population

One hundred forty-four subjects (71 men and 73 women) living in the Tapajós River valley (Brazilian Amazon, State of Pará), aged 15 to 83 yr, were studied. The MeHg exposure in this region is through contaminated fish intake (Passos et al. 2008). All subjects participated in the study on a voluntary basis. Villagers' data were collected using two interviewer-administered questionnaires. One collected sociodemographic information (gender; age; village of residence; place of birth; length of time in the region; educational level; subsistence activities; work in gold mining and exposure to Hg through burning amalgam; exposure to other contaminants; frequency and quantity of smoking, drinking, and drug habits; medical history; and medication). The second was a 7-day recall food consumption frequency questionnaire. For fish consumption, a list was prepared that included most of the fish species present in the region. For each day, participants indicated the number of meals containing fish as well as fish species that were consumed. Anthropometric measurements (weight, height,

and waist circumference) were also taken by a trained technician. Approval was obtained from the Ethics Committee of the University of São Paulo at Ribeirão Preto (Brazil), protocol number CEP/FCFRP-71. The study was explained individually to individuals who agreed to participate, who then signed an informed consent form.

Samples From Blood and Hair and Hg Analyses

Blood samples were collected in trace metal-free evacuated tubes (BD Vacutainer, BD Vacutainer, Franklin Lakes, NJ) containing heparin. Hair samples were obtained from the occipital area of the head, close to the scalp. The lock of hair was stapled at the base and stored in identified Ziploc bags. For the present analysis, the first centimeter of hair scalp was used.

Determination of Hg concentrations in blood and hair was performed as described by Palmer et al. (2006) and Rodrigues et al. (2008), respectively, using inductively coupled-plasma mass spectrometry (ICP-MS) (ELAN DRC II, Perkin Elmer, Norwalk, CT). Results are expressed in micrdograms per liter and micrograms per gram.

Quality control of Hg determination was guaranteed by analyzing standard reference materials from the U.S. National Institute of Standards and Technologies (NIST). Moreover, various secondary reference materials, provided by the New York State Department of Health (NYS DOH PT Program for Trace Elements in Whole Blood and Plasma) and/or by the National Institute of Public Health of Quebec, Canada (INSP External Quality Assessment Scheme [EQAS] for Trace Elements in Blood, Plasma and Hair), were analyzed. Reference samples were analyzed before and after 10 ordinary samples. All results were within the standard values.

Reduced Glutathione (GSH) Levels

Reduced glutathione (GSH) was determined in total blood by addition of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Ellman (1959). DTNB, a symmetric aryl disulfide, reacts with free thiols to form disulfide plus 2-nitro-5-thiobenzoic acid. The product of this reaction is quantified by absorbance at 412 nm. Results are expressed as micromoles per milliliter of total blood.

DNA Isolation and Genotyping

Genomic DNA was extracted from whole blood using the Easy-DNA Kit (Invitrogen, Carlsbad, CA, catalog number K1800-0) according to the manufacturer's instructions and stored at –20°C until analyses.

GSTM1/GSTT1 polymorphisms were genotyped using multiplex PCR as described by Abdel-Rahman et al. (1996). The primers used (Invitrogen, Carlsbad, CA) for *GSTM1* were 5′-GAA CTC CCT GAA AAG CTA AAG C-3′ and 5′-GTT GGG CTC AAA TAT ACG GTG G-3′, generating a 215-base-pair (bp) fragment. For *GSTT1*, the primers used were 5′-TTC CTT ACT GGT CCT CAC ATC TC-3′ and 5′-TCA CCG GAT CAT GGC CAG CA-3′, generating a 480-bp fragment. Further, as an internal control, the CYP1A1 gene (exon 7), which does not present any type of polymorphisms, was also used, and the primers used were 5′-GAA CTG CCA CTT CAG CTG TCT-3′ and 5′-CAG CTG CAT TTG GAA GTG CTC-3′, generating a 312-bp fragment. After amplification, PCR products were subjected to electrophoresis on a 2% agarose gel (Invitrogen, Carlsbad, CA) and visualized using ethidium bromide (Sigma-Aldrich, St. Louis, MO). DNA from samples positive for the *GSTM1* and *GSTT1* genotypes yielded bands of 215 and 480 bp, respectively, while the internal positive control (*CYP1A1*) PCR product corresponded to a 312-bp fragment (Figure 1). Although this technique does not distinguish between the subjects that were heterozygous or homozygous for the positive genotypes, this method conclusively identifies the null genotypes (*GSTM1*0* and *GSTT1*0*).

Statistical Analysis

Descriptive statistics were used to examine the distribution of relevant sociodemographic

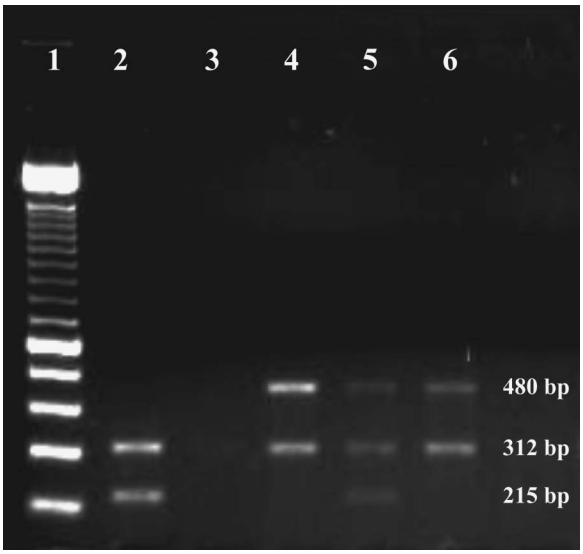

FIGURE 1. PCR profiles in agarose gel (2% w/v) for *GSTM1* and *GSTT1* polymorphisms. 1, Ladder 100 bp; 2, *GSTM1/GSTT1*0*; 3, negative control; 4 and 6, *GSTM1*0/GSTT1*; 5, *GSTM1/GSTT1*.

characteristics and biomarkers of exposure and effect. Simple linear regressions and nonparametric techniques (Spearman's rho) were first used to examine the relations between Hg biomarkers and sociodemographic variables. Linear multiple regression analyses were carried out to examine the associations between GST polymorphisms and B-Hg, H-Hg, and GSH levels. Age and fish consumption were used as continuous variables, while gender, alcohol consumption, and smoking status were included as categorical variables. Results were defined as statistically significant for a value of $p \leq .05$. Analyses were performed using PASW 17 Statistics software (IBM; Armonk, NY).

RESULTS

The sociodemographic characteristics and biochemical measurement of GSH for the 144 participants enrolled in the present study are described in Table 1. The subjects included in the present study were then divided into three groups (B-Hg, H-Hg, and H-Hg/B-Hg ratio), and the multiple regression analyses are summarized in Table 2. Figure 2 illustrates the concentrations of B-Hg and H-Hg among the four possible combinations of genotypes. Results demonstrated that individuals who presented the *GSTM1*0/GSTT1* allele ($n = 36$) showed significantly lower B-Hg and H-Hg levels than individuals with both null genotypes ($n = 25$); for example, the mean B-Hg level for the individuals with the *GSTM1*0/GSTT1* genotype was 37.7 μg/L but was 57.3 μg/L for those who did not present the genotype. Moreover, *GSTM1*0/GSTT1*0* individuals presented higher levels of B-Hg and H-Hg than those with any other genotype. In general,

TABLE 1. Sociodemographic Characteristics of 144 Riparian Studied Individuals of Amazon Region

	n	Percentage (%)	Mean ± SD	Range	Median
Individuals	144	100.0	—	—	—
Genotypes[a]					
GSTM1/GSTT1	51	35.4	—	—	—
*GSTM1/GSTT1*0*	32	22.2	—	—	—
*GSTM1*0/GSTT1*	36	25.0	—	—	—
*GSTM1*0/GSTT1*0*	25	17.4	—	—	—
Age (years)	144	—	43 ± 17	15–83	44.0
Gender	144	—	—		
Men	71	49.3	—	—	—
Women	73	50.7	—	—	—
Fish consumption[b] (portion/d)	—	—	2.5 ± 1.2	1–4	2.0
Body (BMI)	144	—	25.0 ± 4.7	16.4–41.6	24.0
Blood-Hg (B-Hg) (μg/L)	144	—	37.7 ± 24.5	4.7–122.0	32.0
Hair-Hg (H-Hg) (μg/g)	144	—	10.4 ± 7.4	1.0–43.3	8.5
Ratio H-Hg/B-Hg	144	—	297.3 ± 113.7	95.7–623.3	274.1
Gluthatione (μmol/ml erythrocytes)	144	—	1.6 ± 0.5	0.5–2.9	1.63

[a]GSTM1 and GSTT1: wild alleles; GSTM1*0 and GSTT1*0: null alleles.
[b]Portion = 200 g.

TABLE 2. Beta (β) and Significance (*p*) Values From a Multiple Linear Regression Analysis for mercury (Hg) in Blood (B-Hg), Hg in Hair (H-Hg) and the Ratio H-Hg/B-Hg, Using the Genotypes *GSTM1* and *GSTT1* Polymorphisms, Glutathione (GSH) Concentrations, Fish Consumption, Gender and Age as Independent Variables

	B-Hg (37.7 ± 24.5 μg/L)		H-Hg (10.4 ± 7.4 μg/g)		H-Hg/B-Hg (297.3 ± 113.7)	
	β	*p*	β	*p*	β	*P*
Genotypes[a]						
GSTM1/GSTT1	−0.318	<.001	−0.293	<.001	−0.248	.805
*GSTM1/GSTT1*0*	0.123	.151	0.123	.162	−0.003	.976
*GSTM1*0/GSTT1*	0.232	.006	0.205	.018	−0.037	.702
*GSTM1*0/GSTT1*0*	0.458	<.001	0.447	<.001	−0.741	.460
GSH (μmol/ml erythrocytes)	−0.201	.008	−0.139	.073	−0.045	.964
Fish consumption (portions/d)[b]	0.181	.017	0.078	.321	−0.162	.064
Gender	−0.161	.054	0.072	.381	0.158	.088
Age	0.085	.283	0.152	.062	0.086	.346

Note. The individuals ($n = 144$) were divided in four groups according to their genotypes. Hg levels in blood and hair are expressed as mean ± standard deviation.

[a]GSTM1 and GSTT1: wild genotypes; *GSTM1*0 and GSTT1*0*: null genotypes.

[b]One portion is approximately 200 g.

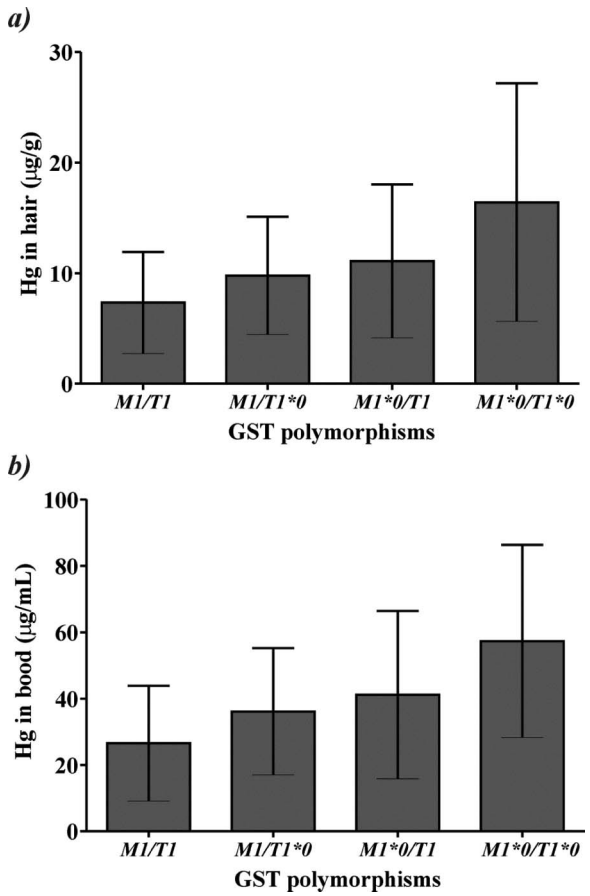

FIGURE 2. Total mercury (Hg) in hair (a) and blood (b) in four different combinations of the genotypes: *GSTM1/GSTT1*, *GSTM1/GSTT1*0*, *GSTM1*0/GSTT1*, and *GSTM1*0/GSTT1*0*.

*GSTM1/GSTT1*0* individuals also presented lower levels of Hg in both hair and blood when compared to the null genotypes, but this difference did not reach statistical significance.

As expected, fish consumption also present influence on the levels of the metal, although interestingly, statistical significance was only found between fish diet and total Hg levels in the bloodstream. Moreover, no significant effects were found with respect to the influence of polymorphisms on GSH levels and therefore, no further conclusions were drawn (data not shown). A negative correlation between GSH in blood and B-Hg and H-Hg levels was also found. In general, individuals with high levels of GSH presented lower concentrations of Hg in the bloodstream and hair (Figure 3).

DISCUSSION

The results of the present study are in agreement with earlier findings that show high levels of Hg exposure in the Amazon region (Dórea et al. 2005, Pinheiro et al. 2006; 2008; Passos et al. 2008). It is notable that high variations in Hg levels were found among participants in the present study; for example, in total blood, Hg levels ranged from 4.7 to 122 μg/L, while in hair the variation was from 1 to

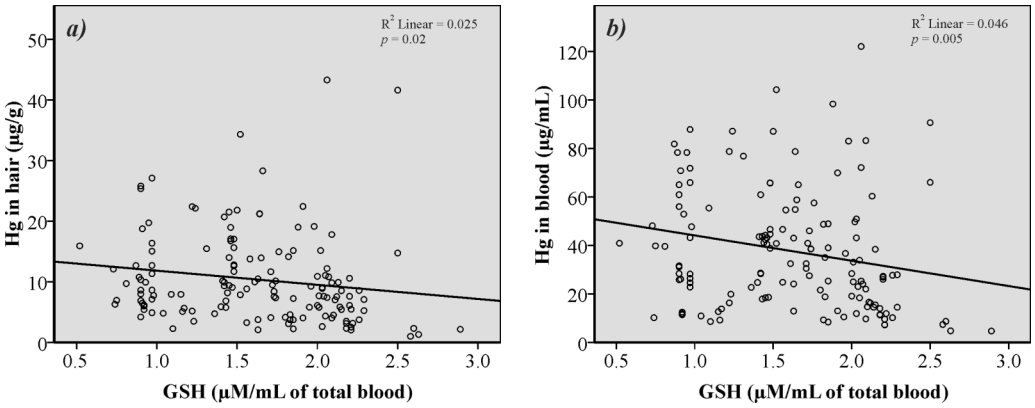

FIGURE 3. Relationship between total mercury (Hg) in hair (a), total Hg in blood (b), and glutathione levels (GSH).

43.3 µg/g. In an earlier study, Passos et al. (2008) demonstrated that fish consumption was related to Hg concentrations in the same population. In the present study, fish intake ranged between one and four portions per day, and this can also be related to variations in metal concentrations in the blood stream and hair. However, fish consumption per se does not completely explain the wide range of Hg levels observed among the subjects of this study. As demonstrated in Table 2, fish consumption only exerts influence on the concentrations of the metal in blood, which reflect an acute exposure. Therefore, the findings provide evidence that other intrinsic factors, such as individual genetic variations associated with MeHg metabolism, may also be related to these discrepancies.

In the present study, 17% of the population presented both null genotypes and 25% and 22% as *GSTM1*0/GSTT1* and *GSTM1/GSTT1*0*, respectively. Approximately 36% of subjects had both genes without the polymorphic deletion. Rossini et al. (2002) studied 591 volunteers from Rio de Janeiro, Brazil, and found frequencies of 42.1 and 25.4% for *GSTM1*0* and *GSTT1*0* polymorphisms, values higher than those detected in the present study. Some epidemiological studies demonstrated differences among people from different ethnic groups, for example, Africans, Asians, and Afro-Americans (Mo et al. 2009). The population in this study is mainly traditional or *Cabocla*, originating from the miscegenation of indigenous populations with European colonizers and to a lesser extent African slaves during the 18th and the 19th centuries (Lima 1992; Murrieta 2001), and also includes migrants mainly from the northeast of Brazil who came to the region in the 1960s and 1980s (Carvalho 1982). Therefore, comparison with other epidemiological data can be misleading.

To our knowledge, this is the first study to evaluate the influence of *GSTM1* and *GSTT1* polymorphisms on a population chronically MeHg exposed via consumption of contaminated fish. Other studies concerning these polymorphisms were carried out; however, the populations of those studies did not reflect the same exposure situation. For example, while our group displayed blood Hg values higher than 100 µg/L (mean = 37.7 µg/L), Gundacker et al. (2007) noted bloodstream Hg values ranging between 0.11 and 7.79 µg/L (mean = 1.73 µg/L), approximately 22-fold lower than the levels found in the population of the present study.

As indicated in the Results section, individuals who carry the *GSTT1*0*, *GSTM1*0*, or the combination of both genotypes present clearly higher B-Hg and H-Hg levels than those who carry the wild genotypes. Our data corroborate the findings of Lee et al. (2010), who evaluated 417 North Korean pregnant women exposed to MeHg via consumption of contaminated fish and demonstrated that women with the *GSTM1*0* and *GSTT1*0* polymorphisms presented higher Hg concentrations in both maternal blood and in umbilical cord blood of

respective newborns. Klautau-Guimarães et al. (2005) studied an Amazon Amerindian population and found the same correlation with individuals who carry null genotypes of *GSTM1* presenting higher B-Hg concentrations than those who carry the wild genotype. Therefore, data suggest that genetic factors may be used as an indicator of effect on Hg metabolism. However, there is no information concerning the relationship between health status of the studied individuals and polymorphic genotypes, and for this reason, additional conclusions can not be drawn. Further epidemiological studies are necessary to have a better elucidation of the implications of these polymorphisms on the Amazon communities health conditions.

In studies of Swedish students, Custodio et al. (2004; 2005) determined the polymorphisms of *GSTA1*, *GSTP1*, *GSTM1*, and *GSTT1* associated with Hg metabolism. Results showed that *GSTM1*0* and *GSTT1*0* polymorphisms exerted no effect on either inorganic Hg or MeHg levels; however, the combined effects of *GSTT1*0* and *GSTM1*0* polymorphisms was not examined. In these studies, the only relationship noted was between *GSTP1* polymorphisms and Hg metabolism. Engström et al. (2008) also demonstrated that polymorphisms of *GSTP1* are associated with MeHg retention in the same population. These differences that were found between these earlier studies and the present investigation can be partly explained by the variation in ethnicity, where the Swedish population is composed almost exclusively by Caucasian individuals, while Amazon populations present a high ethnic variation. Moreover, some studies showed that homozygous deletion in *GSTM1* and *GSTT1* increases the sensitization and neurotoxicity associated with exposure to other mercurial compounds such as thimerosal (ethylmercury), which is used as a preservative in vaccines and ophthalmological preparations (Westphal et al. 2000). In contrast, Alinovi et al. (2002) investigated biomarkers related to renal system damage and dysfunctions in subjects exposed to low doses of Hg from different sources and found no marked influence of *GSTM1* and *GSTT1* genotypes on urinary Hg excretion in either control or exposed workers.

In a study carried out in the Amazon region, Pinheiro et al. (2008) observed a negative correlation between chronic Hg exposure and biochemical parameters related to redox status (activity of the antioxidant enzyme catalase and levels of the tripeptide GSH). A negative association between B-Hg and H-Hg levels and total GSH was also noted in this study. Further, Grotto et al. (2010) demonstrated a correlation between MeHg levels and disturbances in redox status, such as inverse relations among glutathione peroxidase, delta-aminolevulinic acid dehydratase, catalase, and GSH, with B-Hg and H-Hg in the same population as in the present study.

In conclusion, the present data show that Hg levels in total blood and hair of Amazon communities display wide individual variation. Our findings demonstrated that genetic variations may be related to Hg metabolism, given that the combination of *GSTM1*0* and *GSTT1*0* genotypes may explain the extensive differences in B-Hg and H-Hg levels. Moreover, individuals who carry only the *GSTT1* wild genotype (*GSTM1*0/GSTT1*) also present lower concentrations of Hg, suggesting that the *GSTT1* genes play an important role in Hg metabolism in this population, since reduced enzyme activity may reduce Hg excretion via Hg-GSH conjugates, raising Hg retention in the body, while no significant correlation was noted between GSH levels and GST polymorphisms, suggesting that other intrinsic factors may influence this phenomenon. Therefore, further studies concerning other polymorphisms in different detoxification steps need to be considered for a better understanding of the mechanisms involved in metal toxicity and excretion.

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