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Mercury Levels and Glutathione S-Transferase Polymorphisms Evaluation in a Population of the Low Amazon, Brazil

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Abstract

Mercury (Hg) is considered one of the most dangerous toxic metals to the environment and to human. High Hg level may induce different types of toxic effects on human health and the main target is the Central Nervous System (CNS). One of the main mechanisms involved in neurotoxicity caused by Hg is the oxidative stress and its effect on antioxidant systems. Currently it is known that deforestation is one of the main reasons for the high levels of Hg in the aquatic ecosystems in Amazon. However, to the best of our knowledge, there are no recent investigations that evaluate the Hg exposure in a human population of the Low Amazon region. The present work aims to fill the gap of the lack of information related to how the urban and floodplain populations of Santarém city (Pará state, Brazilian Amazon) is affected by Hg exposure, also investigating the *GSTM1* and *GSTT1* polymorphisms for the population studied. Our results show that the individuals studied are environmentally exposed to Hg due to the fish intake. Additionally, the analysis of our data suggests that fish intake, age, and sex are more relevant for prediction of Hg level in blood than the polymorphisms of *GSTM1* and *GSTT1* genes.

Introduction

Mercury (Hg) is one of the most dangerous heavy metal due to its high toxicity, bioaccumulative properties and genotoxic potential, with harmful effects for the environment and for human health [1]. Hg is released in the environment from natural sources and/or anthropic activities and circulates between the atmosphere and aquatic and terrestrial ecosystems, potentially reaching humans [2].

In the Tapajós River, the human exposition to the Hg has been strongly related to the consumption of contaminated fishes [3]. The presence of Hg in fishes and humans was firstly reported in the Amazon in the early 1990 [4]. Since then, many of the data on the cumulative Hg levels in fish and human populations in this region focus on gold digging activity (*garimpo*) as the main responsible for the Hg environmental contamination [5], although less than 3% of the Hg deposited in Amazonian soils comes from the *garimpo* [6].

Currently, a major contributor to the maintenance of high levels of Hg in the Tapajós River comes from the illegal deforestation caused by the expansion of the agricultural border [7]. The conversion of forest soil in pasture soil through the burning increases the remobilization of Hg accumulated [8] and intensifies the erosion process that accelerates the transport of Hg for aquatic environments [9]. Once there, it can be biotransformed into methylmercury (MeHg), the most toxic Hg compound to humans. The MeHg is neurotoxic and crosses the placental and the blood-brain barriers and may result in different types of fetal abnormalities (neurological and motor) [10]. Furthermore, low levels of MeHg are sufficient to cause adverse toxic effects in adults and infants [11].

One of the key mechanisms involved in neurotoxicity caused by MeHg is oxidative stress and its effect on antioxidant systems [12] especially the Glutathione (GSH) [13], an intracellular antioxidant

that reacts with MeHg forming the MeHg-glutathione complex, an essential step in Hg detoxification. Glutathione S-Transferase (GST) is a family of highly polymorphic enzymes that catalyze the MeHg-glutathione complex conjugation reaction or may act the transporters of MeHg-glutathione complexes [14]. Recent epidemiological studies link polymorphism alteration of GST genes to Hg levels [15-18]. *GSTM1* and *GSTT1* deletions were associated with high levels of Hg in Amazon riverine [19] and indigenous [20] populations.

Thus, the aim of the present work is to investigate the environmental exposure to Hg in the urban and floodplain populations of Santarém city (Low Amazon region) evaluating the total Hg level, in association to factors such as age, sex, and the frequency of fish intake, as well as the polymorphisms of oxidative stress-related genes (*GSTM1* and *GSTT1*).

Methodology

An observational, descriptive and quantitative study was conducted. The study enrolled 98 subjects of both sexes, aged between 18 and 81 years living in the Santarém urban area (n = 63), located at the confluence of Tapajos and Amazonas rivers and in the Santarém *várzea* area (n=35), located in the floodplain on opposite banks of the Amazon river. Both groups are formed by residents of the Santarém municipality region, which belongs to the middle region of the low Amazon. The study was approved by the Ethics Committee of Pará State University (UEPA) (technical report no. 1,127,108).

All participants were previously informed about the objectives of the study and a health/lifestyle questionnaire after the signature of a consent term. Blood samples were collected in tubes containing EDTA and total Hg blood concentration was determined by flameless atomic absorption spectrometry in DMA-80 Direct Mercury Analyzer (Milestone) equipment, according to the manufacturer's instructions, and for each sample, the analyses were made in duplicates. According to WHO, those individuals who have Hg levels above 10μg/L were considered exposed.

Genomic DNA was extracted from blood samples of all subjects using the *QIAamp DNA Blood Mini Kit* (Qiagen), according to the manufacturer's instructions and stored at -20 ˚C until analysis. Genotyping was performed by PCR multiplex for *GSTM1* and *GSTT1* genes. The PCR was carried out in a total volume of 20 μL, containing 3 μL of DNA template (20-50ng), 1 μL of each primers (10mM), 10 μL of PCR Master Mix (Promega) (50 units/mL of Taq DNA Polymerase supplied in a proprietary reaction buffer (pH 8.5), 400 μM each dNTP, 3 μM MgCl2) and 1 μL of Nuclease-free water.

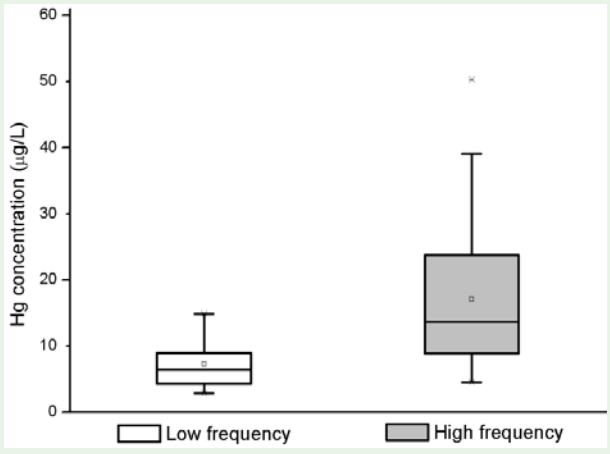

Figure 1: Relationship between Hg levels and frequency of fish intake.

The primers used were *GSTM1* Forward *5'-GAACTCCCTGAAAAGCTAAAGC-3'* and Reverse *5'-GTTGGGCTCAAATATACGGTGG-3'* and the *GSTT1* primers Forward *5'- TCACCGGATCATGGCCAGCA-3'* and Reverse *5'-TTCCTTACTGGTCCTCACATCTC-3'*. As an internal control *β-globin* gene was co-amplified using the primers forward *5'-GAAGAGCCAAGGACAGGTAC-3'* (GH20) and Reverse *5'-CAACTTCATCCACGTTCACC-3'* (PC04). The reaction was incubated at 94 ˚C for 5 min, prior to 35 cycles of denaturation of 1 min at 94 ˚C, annealing of 1 min at 65 ˚C, and extension of 1 min at 72 ˚C, followed by a final extension of 7 min at 72 ˚C. The PCR products were visualized by electrophoresis in 3% Agarose gel. The internal control fragment amplified from *β-globin* was 268 bp of length and the normal (presence) or deletion (absence) of *GSTM1* and *GSTT1* genes was detected by 215 and 459 bp fragments, respectively.

The contributions of the present work are twofold. First, the relationship between levels of Hg and frequency of fish intake was investigated using the Kruskal-Wallis test, in order to verify if individuals with higher frequency of fish intake have higher Hg levels. Second, a multiple regression analysis was performed in order to investigate the effects on Hg levels due to the variables age, fish intake, sex, and presence of *GSTM1* and *GSTT1* polymorphisms. In relation to fish consumption, the sample was stratified into two subgroups: the high-frequency subgroup, with a consumption of fish daily or weekly; and the low frequency subgroup with a biweekly or monthly consumption. Statistical tests were performed in STATA 7.0 software, with a 5% significance level. The graph was constructed using ORIGIN 8.0 software.

Results

The study included 98 individuals of which 63 (64.3%) live in urban area and 35 (35.7%) in floodplain area of Santarém. Among them, 69.4% were female and 30.6% male, most aging between 21 and 40 years (59.8%). The average levels of total Hg were measured in the blood of the 98 subjects studied and it was observed that 54.1% had levels above the recommended by the WHO. Table 1 shows the descriptive statistics about Hg level in both health and lifestyle of the analyzed subgroups.

It was verified the *GSTM1* and *GSTT1* genotypes frequencies in the 98 individuals studied (Table 2). The genotypes of 95 individuals for the *GSTM1* polymorphism were obtained successfully, among them 69.5% were normal and 30.5% were deletion. Regarding the *GSTT1* polymorphism, 98 genotypes were obtained successfully, of which 84.7% were normal and 15.3% were deletion. The Pearson Chi-Square statistics as well as the p-values reported in Table 2 indicate that the hypothesis of independence between the table variables cannot be rejected, therefore with no significant association between them.

In order to verify the relationship between Hg levels and fish intake, the sample was stratified into two groups, namely: high frequency (n = 66), with a daily or weekly intake; and low frequency (n = 32) with a biweekly or monthly intake. Kruskal-Wallis test was used to compare the total Hg levels among subgroups of high and

Table 1: Health/Lifestyle Characteristics and Hg levels of the individuals analyzed.

Health/Lifestyle Characteristics	n	%	Levels of Hg in blood (µg/L)				
			Mean (µg/L)	Standard Deviation	Median (µg/L)	Maximum (µg/L)	Minimum (µg/L)
Age*							
18-20 years	8	8.3	7.1	2.8	6.7	12.3	3.2
21 -40 years	58	59.8	12.0	8.1	9.9	37.3	2.9
41 – 60 years	26	26.8	19.3	11.7	15.2	50.3	4.1
More 60 years	5	5.1	18.9	13.1	17.4	39.0	7.3
Sex							
Male	30	30.6	16.6	12.2	12.7	50.3	2.9
Female	68	69.4	12.7	8.4	10.3	39.1	3.7
Hg levels							
Until10µg/L	45	45.9	6.6	1.9	6.8	9.7	2.9
Above 10µg/L	53	54.1	20.0	9.7	15.6	50.3	10.2
Fish intake							
High frequency	66	67.3	17.1	10.4	13.7	50.3	4.5
Low frequency	32	32.7	7.3	3.2	6.6	14.9	2.9
Domicile							
Urban area	63	64.3	8.9	4.4	8.3	25.9	2.9
Floodplain area	35	35.7	22.8	10.6	23.0	50.3	7.7

*The sum of subjects in all age subgroups was 97 because information was missing during the questionnaires application.

Table 2: GSTM1 and GSTT1 polymorphisms genotypes frequencies of the individuals analyzed.

Health/Lifestyle Characteristics	*GSTM1* Genotypes (n=95)				*GSTT1* Genotypes (n=98)			
	Normal (n=66)	Deletion (n=29)	x^2	*p*	Normal (n=83)	Deletion (n=15)	x^2	*p*
Age			4.06	0.25			0.94	0.81
18-20 years	06 (9.1%)	02 (7.2%)			06 (7.3%)	02 (13.3%)		
21 - 40 years	37 (56%)	21 (75%)			49 (59.8%)	09 (60%)		
41 - 60 years	18 (27.3%)	05 (17.8%)			23 (28%)	03 (20%)		
More 60 years	05 (7.6%)	0			04 (4.9%)	01 (6.7%)		
Sex			0.31	0.58			0.73	0.39
Male	19 (28.8%)	10 (34.5%)			24 (28.9)	06 (40%)		
Female	47 (71.2%)	19 (65.5%)			59 (71.1%)	09 (60%)		
Hg levels			2.12	0.14			0.004	0.95
Until10µg/L	28 (42.4%)	17 (58.6%)			38 (45.8%)	07 (46.7%)		
Above 10µg/L	38 (57.6%)	12 (41.4%)			45 (54.2%)	08 (53.3%)		
Fish intake			0.34	0.56			0.004	0.95
High frequency	45 (68.2%)	18 (62.1%)			56 (67.5%)	10 (66.7%)		
Low frequency	21 (31.8%)	11 (37.9%)			27 (32.5%)	05 (33.3%)		
Domicile			0.13	0.71			0.44	0.83
Urban area	43 (65.2%)	20 (69%)			53 (63.8%)	10 (66.7%)		
Floodplain area	23 (34.8%)	09 (31%)			30 (36.2%)	05 (33.3%)		

Table 3: Hg levels and *GSTM1* polymorphism genotypes in the individuals analyzed.

GSTM1 Genotypes	n	Hg Levels - High Frequency subgroup								
		Until 10µg/L	Above 10µg/L	Mean (µg/L)	Standard Deviation	Median (µg/L)	Maximum (µg/L)	Minimum (µg/L)	x^2	*p*
Normal	45	12(26.7%)	33(73.3%)	18.2	11.1	15.3	50.3	4.5	1.87	0.17
Deletion	18	08(44.4%)	10(55.6%)	14.1	8.1	11.0	32.1	4.8		
Total	63									
***GSTM1* Genotypes**	**n**	**Hg Levels – Low Frequency subgroup**								
		Until 10µg/L	Above 10 µg/L	Mean (µg/L)	Standard Deviation	Median (µg/L)	Maximum (µg/L)	Minimum (µg/L)	x^2	*p*
Normal	21	16(76.2%)	05(23.8%)	7.5	3.5	6.1	14.9	3.2	0.13	0.71
Deletion	11	09(81.8%)	02(18.2%)	6.9	2.8	6.7	12.3	2.9		
Total	32									

Table 4: Hg levels and *GSTT1* polymorphism genotypes in the individuals analyzed.

GSTT1 Genotypes	n	Hg Levels - High Frequency subgroup								
		Until 10μg/L	Above 10μg/L	Mean (μg/L)	Standard Deviation	Median (μg/L)	Maximum (μg/L)	Minimum (μg/L)	x^2	p
Normal	56	18(32.2%)	38(67.8%)	17.7	10.9	13.8	50.3	4.5	0.0005	0.98
Deletion	10	03(30%)	07(70%)	13.9	6.4	11.5	27.0	6.9		
Total	66									
***GSTT1* Genotypes**	**n**	**Hg Levels – Low Frequency subgroup**								
		Until 10μg/L	**Above 10 μg/L**	**Mean (μg/L)**	**Standard Deviation**	**Median (μg/L)**	**Maximum (μg/L)**	**Minimum (μg/L)**	x^2	p
Normal	27	21(77.8%)	06(22.2%)	7.3	3.2	6.4	14.9	2.9	0.012	0.91
Deletion	05	04(80%)	01(20%)	7.4	3.5	6.8	12.3	3.2		
Total	32									

Table 5: Univariate and multivariate regression coefficients for the response variable Hg level.

Variable	Univariate Analysis			Multivariate Analysis (R^2 = 0.3717)	
	β	p	R^2	β_i	p
Fish Intake	9.790	<0.005	0.2207	8.547	<0.005
Age	0.307	<0.005	0.1696	0.231	0.001
Sex	3.891	0.071	0.0337	3.869	0.036
GSTM1	3.384	0.122	0.0255	1.335	0.478
GSTT1	2.582	0.351	0.0090	2.452	0.301

low frequency. The results demonstrated that individuals of the high frequency subgroup often have total Hg levels higher compared to the low frequency subgroup (Kruskal-Wallis test, $\chi^2 = 30.167$, p = 0.0001) (see Figure 1. The asterisk in the figure corresponds to an outlier for the high frequency subgroup).

Tables 3 and 4 exhibit Hg levels for *GSTM1* and *GSTT1* polymorphisms respectively. Regarding the *GSTM1* polymorphism, most individuals of both high and low frequency subgroups of fish intake have the normal gene, with mean Hg levels of 18.2 μg/L and 7.5 μg/L, respectively. The hypothesis of independence of variables cannot be rejected for the individuals studied, therefore with no significant association between *GSTM1* polymorphism genotypes and Hg level in high ($\chi^2 = 1.87$, p = 0.17) and low frequency ($\chi^2 = 0.13$, p = 0.71) subgroups. Regarding the *GSTT1* polymorphism, in both subgroups the normal gene was more frequent, with mean levels of total Hg 17.6 μg/L in the high frequency subgroup and 7.3 μg/L, the low frequency subgroup. Again, the hypothesis of independence of variables cannot be rejected for the individuals studied, therefore with no significant association between *GSTT1* polymorphism genotypes and Hg level in ($\chi^2 = 0.0005$, p = 0.98) and low frequency ($\chi^2 = 0.012$, p = 0.91) subgroups.

A multiple linear regressions was run to predict Hg level from the variables fish intake, age, sex, presence of *GSTM1* polymorphism, and presence of *GSTT1* polymorphism. Table 5 exhibits both univariate and multivariate regression coefficients.

Discussion

Epidemiological studies are important tools to assess the relationship between exposure and their respective toxic effects and thus characterize the risk to human and environmental health [2]. The assessment of the risks to human health from exposure to Hg in the Amazon region has focused mainly on enviromental factors [21]. However, it has been shown that genetic factors may influence toxicokinetics and/or toxicodynamics of Hg [22]. Therefore, the present study evaluated the total Hg levels in the blood of individuals living in Santarém in order to investigate the association with the consumption of fish and/or the absence of the *GSTM1* and *GSTT1* polymorphisms involved in the MeHg-glutathione complex conjugation reaction and/or transporter.

Different studies have been performed with riverine populations of the Tapajós River to assess whether the Hg levels are associated with frequent fish intake [21]. In the present study, the average levels of total Hg found in the blood was 13.9 μg/L, which corroborates the hypothesis that the population of Santarém has shown Hg levels above the limit recommended by the WHO [23] over the years. Our results showed that subjects from the high frequency fish intake subgroup have higher levels of Hg compared to subgroup of low frequency ($\chi^2 = 41.68$; p = 0.0001), both in urban and floodplain areas. Then, we conclude that the population of Santarém is environmentally exposed to Hg poisoning through frequent fish intake.

Regarding effects on the Hg level in blood, the variables fish intake, age, sex, *GSTM1* polymorphism, and *GSTT1* polymorphism (Table 5) statistically significantly predicted Hg level, F(5, 88) = 10.41, p < 0.0005, $R^2 = 0.3717$. The variables fish intake, age and sex added statistically significantly to the prediction (p < 0.05), although the presence or deletion of *GSTM1* or *GSTT1* polymorphisms did not (p = 0.478 and p = 0.301, respectively).

Conclusion

From the blood levels assessment of total Hg in the present study, it can be concluded that urban and floodplain populations of Santarém are environmentally exposed to Hg contamination through frequent fish intake. According to the analysis of our data, the fish intake, age and sex are more relevant for the Hg level in blood than genetic factors related to *GSTM1* and *GSTT1* polymorphisms. In a near future, the population of Santarém will be exposed to new environmental impacts of deforestation growth and installation of complex hydropower plants at Tapajós River. We highlight the need of

further studies in order to determine which environmental factors (in addition to the frequent fish intake) may influence the toxicokinetics and/or toxicodynamics of Hg in humans, as well as genetic factors, in order to minimize damage of Hg exposure to human health.

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