Chemosphere 100 (2014) 89–96

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Iron status as a covariate in methylmercury-associated neurotoxicity risk ☆,☆☆

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HIGHLIGHTS

- Iron stores and methymercury exposure probably occur independently in the Amazon.
- Iron status is unassociated with hair-Hg concentration among high fish eaters.
- Iron status is not a confounder of methylmercury among Amazonian riparians.
- Methylmercury exposure is unrelated with iron stores among young Amazonian females.

ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form 16 December 2013
Accepted 19 December 2013
Available online 8 January 2014

tile range

Keywords:
Iron stores
Mercury
Negative confounding
Fertile women
Amazon
Fish consumption

ABSTRACT

Intrauterine methylmercury exposure and prenatal iron deficiency negatively affect offspring's brain development. Since fish is a major source of both methylmercury and iron, occurrence of negative confounding may affect the interpretation of studies concerning cognition. We assessed relationships between methylmercury exposure and iron-status in childbearing females from a population naturally exposed to methylmercury through fish intake (Amazon). We concluded a census (refuse <20%) collecting samples from 274 healthy females (12–49 years) for hair-mercury determination and assessed iron-status through red cell tests and determination of serum ferritin and iron. Reactive C protein and thyroid hormones was used for excluding inflammation and severe thyroid dysfunctions that could affect results. We assessed the association between iron-status and hair-mercury by bivariate correlation analysis and also by different multivariate models: linear regression (to check trends); hierarchical agglomerative clustering method (groups of variables correlated with each other); and factor analysis (to examine redundancy or duplication from a set of correlated variables). Hair-mercury correlated weakly with mean corpuscular volume ($r = .141$; $P = .020$) and corpuscular hemoglobin ($r = .132$; .029), but not with the best biomarker of iron-status, ferritin ($r = .037$; $P = .545$). In the linear regression analysis, methylmercury exposure showed weak association with age-adjusted ferritin; age had a significant coefficient (Beta = .015; 95% CI: .003–.027; $P = .016$) but ferritin did not (Beta = .034; 95% CI: −.147 to .216; $P = .711$). In the hierarchical agglomerative clustering method, hair-mercury and iron-status showed the smallest similarities. Regarding factor analysis, iron-status and hair-mercury loaded different uncorrelated components. We concluded that iron-status and methylmercury exposure probably occur in an independent way.

Abbreviations: RBC, red blood cells count (10^6 μL^{-1}); MCV, mean corpuscular volume (fL); MCH, mean corpuscular hemoglobin (pg); MCHC, mean corpuscular hemoglobin concentrations (g dL^{-1}); Hair-mercury, total mercury concentration in hair (ppm = $\mu g\ g^{-1}$); Resex, extractive reserve; IQR, interquartile range.

☆ Financial support: Study was supported by INOVA-ENSP Program and CNPq/MCT Universal 478085/2010-9 under the scope of Hydrobiogeochemistry Monitoring Program from Santo Antônio Energia. Marlon de Freitas Fonseca had financial support from CAPES Foundation, Ministry of Education of Brazil, BEX 6186-12-2 as Visiting Scientist by EOME Program in Harvard School of Public Health, Boston, USA.

☆ The research protocol, survey instruments and procedures were reviewed and approved by a Brazilian Research Ethics Committee (CAAE: 0010.0.047.000-09).

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http://dx.doi.org/10.1016/j.chemosphere.2013.12.053

1. Introduction

Fish is the main natural source of methylmercury (MeHg) for humans IPCS, 1990; U.S. Environmental Protection Agency, 2013, but also one of the main sources of iron (WHO, 2001) and other essential nutrients. Mercury and iron stores may antagonistically affect cognition, but most clinical studies that addressed effects of intrauterine MeHg exposure have ignored the potential negative confounding by maternal iron stores (and vice versa). Actually, a possible confounding role of maternal nutrition in studies examining associations between prenatal fish–MeHg exposures and developmental outcomes in children has been suggested (Davidson et al., 2008), whereas a substantial underestimation of the effects of MeHg toxicity and fish benefits may occur from the lack of confounder adjustment (Choi et al., 2008).

Unknown interactions between maternal iron stores and MeHg exposure may actually exist so that some confounding might have occurred and affected prior studies concerning neurodevelopment outcomes as consequence of intrauterine MeHg exposure. In addition to noise from many covariates, there is also a concern about the precision of the exposure assessment, which may cause significant bias from the underestimation of dose-related toxicity (Grandjean and Budtz-Jørgensen, 2010; Grandjean and Herz, 2011; Karagas et al., 2012). In fact, low iron stores increase animal gastrointestinal absorption of iron and of certain trace elements (Pollack et al., 1965) and a strong positive correlation between iron status and serum mercury concentrations was recently found in humans (Bárány et al., 2005).

In Amazon, neurotoxic effects of intrauterine methylmercury exposure have been a concern due to the typical strong nutritional dependence of fish intake (Hacon et al., 2000, 2008). Since both intrauterine MeHg exposure (Grandjean and Herz, 2011) and prenatal iron deficiency (Beard, 2003) negatively affect offspring's brain development, this study aimed to assess the relationship between MeHg exposure and iron status in childbearing females within the specific geographical context of the typical Amazonian riparians living in Madeira River Basin: a population naturally exposed to MeHg, iron and endemic parasitic diseases (Malm et al., 1995; Hacon et al., 2000, 2008; Fonseca, 2007; Passos and Mergler, 2008).

2. Subjects and methods

2.1. Study area and population

The study area is located in the municipality of Porto Velho (8°47′31″S and 63°57′7″W) around Madeira River, the second largest river of the Amazon Basin that transports one of the largest loads of fluvial sediment in the world (Latrubesse et al., 2005). This cross-sectional observational study was performed from May 2009 to April 2011 and included individuals living in regions extending from about 80 km upstream and 180 km downstream of the Santo Antônio rapids (Fig. 1). Villages around Santo Antônio rapids were grouped in 5 different areas in relation to the left and right banks of the river as well as spatial considerations regarding upstream and downstream the rapids. Clusters were chosen in order to consider the possibility of urban–rural differences in food habit (Neuman et al., 2013) and in MeHg exposure, since lowest fish consumption has been found near urban areas (Dutra et al., 2012) and geographical isolation may be a major variable in studies concerning MeHg effects on humans (Fonseca, 2008).

Area 1 (the closest to Porto Velho city) is located at the right bank downstream the falls with access by roads, 7–10 km away from the urban area at the right bank of Madeira River. Despite close to Madeira River, the main source of fish for inhabitants of Area 1 has been a creek (Belmont igarapé). Area 2 (left bank, downstream the falls) comprises villages close to the city of Porto Velho by boat (8–15 km away from the urban area). The main source of fish has been Madeira River channel. Area 3 (upstream the falls at the right bank) comprises relatively isolated villages with regular access to the large carnivorous fish species from Madeira River channel; local people have some access to a major highway and to services and goods available in the capital city of Porto Velho. Area 4 (upstream the falls at the left bank) is another relatively isolated group that included families living up to 80 km from Porto Velho city; specific availability of fish is quite similar to Area 3. Area 5 included the most geographically isolated community among all assessed village groups, with no means of regular transportation in the middle of the forest with no sewer system or safe drinking water supply. It takes place in the extractive reserve (Resex) of the Cuniã Lake (total area of 55 850 ha and total estimated population of 309 individuals), located in the municipality of Porto Velho, on the left side of Madeira River, about 180 km downstream the urban area. The Area 5 (Resex Cuniã) was chosen as a reference area since it reflects the typical life style of several isolated riparian populations. The main source of fish in Resex Cuniã has been the central lake, including small creeks that link it to the Madeira River channel. There is some local primary-health-care only in Areas 2 and 3.

All residents of the areas described were invited to participate in the study (a census); the inclusion criterion was females aging 12–49 years who had lived in these communities for at least one year.

All the adults of each household in the studied areas answered a semi-structured questionnaire (interview) with information on socioeconomic status, demographic, life style, occupational history and self-reported morbidity. The objective dietary question *How often do you eat fish weekly?* was asked for each of the family members. Based on estimated number of residents (previous family enrollment), acceptance rate was 90% in the areas 1–4 and 81% in Area 5 (Resex Cuniã), which was regarded as a good result.

2.2. Biological samples

On the day of the interview, a sample of hair was collected from the occipital area near to the scalp of each participant. Total mercury concentration in individual hair samples (Hair-mercury) was determined according to routine laboratory procedures at the Wolfgang Christian Pfeiffer Environmental Biogeochemical Laboratory (BIOGEOQ) in Federal University of Rondônia (UNIR) and at the Chemistry Department of Pontifical Catholic University of Rio de Janeiro (PUC-Rio). Briefly, after mineralization in acid-oxidant medium, total mercury determination was performed by cold vapor atomic absorption spectrometry in a FIMS-400® Perkin–Elmer (Bastos et al., 1998). All analytical runs included material certified by the International Atomic Energy Agency (IAEA-085 and IAEA-086) and National Research Council Canada (DORM-2). Recovery rates were >80% and detection limit <0.03 mg kg^{-1}.

Blood sampling and analysis were performed by the laboratory of the Nove de Julho Hospital (CEACLIN®), which is located in the city of Porto Velho. Samples were collected in campaigns (visits to the communities) with 10 mL syringe with 25 × 7 BD® disposable needles, transferred to respective collection tubes (BD® Vacutainer) and immediately taken to the laboratory under adequate conditions for avoiding hemolysis. Blood cell tests assessing blood hemoglobin concentration, hematocrit, red blood cells count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)

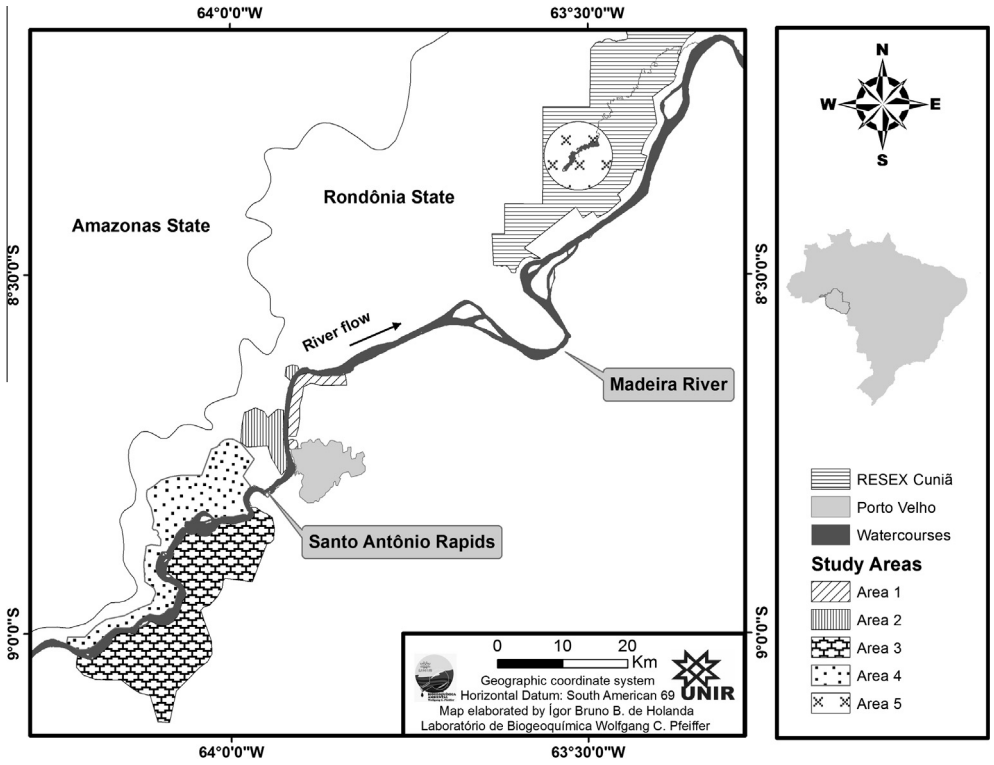

Fig. 1. The study areas: Area 1 (downstream the rapids, right bank), Area 2 (downstream the rapids, left bank), Area 3 (upstream the rapids, right bank), Area 4 (upstream the rapids, left bank) and the extractive reserve of the Cuniã Lake (Area 5), about 180 km downstream the rapids. Porto Velho city is the capital of Rondônia State.

were performed with a 28 parameters Abbott Cell-Dyn 3500® Hematology Analyzer (IL, USA) followed by a classic blood film stain (Giemsa) for peripheral blood smears in a Nikon Eclipse® microscope (NY, USA). Abnormal results were confirmed with a Sysmex KX-21N® Automated Hematology Analyzer (Kobe, Japan). Determinations of serum iron were performed with a Spectrophotometer Roche Hitachi 911® Chemistry Analyzer (IN, USA); Labtest® kits were used for calibration (MO, USA). Results above two standards deviations were checked with a Roche Cobas Mira Plus® Chemistry Analyzer (West Sussex, UK). Qualitative and semiquantitative detection of reactive c protein (RCP) was assessed by agglutination evaluation (reference value = 6 mg L^{-1}); abnormal results were confirmed through immunoturbidimetric assay in a Roche Cobas Mira Plus® Chemistry Analyzer (West Sussex, UK). Serum ferritin, thyroid stimulating hormone (TSH) and thyroxine (T4) concentration was obtained by microparticle enzyme immunoassay (MEIA) in an automatic chemiluminescence analyzer IMMULITE® 1000 System (Munich, Germany). A regular intercalibration program with certified samples has ensured quality control in CEACLIN® laboratory.

We excluded individuals using any iron medication in the last 30 d ($N = 4$), self-reported rheumatoid arthritis ($N = 3$) or hysterectomized ($N = 4$). Because ferritin is an acute-phase protein, RCP values were used to identify individuals whose serum ferritin concentration could be elevated as a response to a major infection or inflammation; exclusion criteria were RCP $\geqslant$ 6 mg L^{-1} ($N = 23$). Because extreme alterations in the thyroid function could be a problem in serum ferritin interpretation, thyroid stimulating hormone (TSH) and thyroxine (T4) concentration were also assessed in order to exclude severe cases. There were ten individuals with insufficient sampled hair mass who were not included in the study. Finally, we considered 274 healthy females residing near the Madeira River channel (Areas 1–4) or in the lacustrine environment of Resex Cuniã (Area 5).

The research protocol, survey instruments and procedures were reviewed and approved by a Brazilian Research Ethics Committee (CAAE: 0010.0.047.000–09); consent forms were obtained from the participants after comprehensive explanation.

2.3. Statistics

Database was managed using Microsoft Office Acces® (version 2010; Microsoft Corp, Redmond, WA, USA). Graphics and statistics were performed with IBM® SPSS® Statistics Standard Grad Pack 20 (NY, USA). Statistical results were considered significant when $P < 0.05$ (2-sided). Overall, characteristics of subjects in the study areas were compared through nonparametric test (independent samples). Although the central limit theorem states that, even if a population distribution is strongly non-normal, it is conceivable to use parametric statistic for assessing sufficiently large samples, the data distribution of all variables (histogram) were evaluated and, besides, a Kolmogorov–Smirnov normality test was used before the exploratory parametric statistical analysis. The two non-normally distributed variables (hair mercury and serum ferritin concentrations) were then Ln-normalized because their exponential pattern of distribution. Associations between MeHg exposure (Hair-mercury) and the main clinically used variables for iron status quantification were first assessed by bivariate correlations and, then, by different multivariate analysis. A multiple linear regression model was used to assess the relationship between MeHg exposure (Hair-mercury, dependent variable) as a function of the main biomarker and best isolated predictor of body iron stores (serum ferritin concentration); a hierarchical agglomerative clustering method (with basis on squared Euclidean distance) was used to identify groups of variables correlated with each other; and a factor analysis (principal components; orthogonal rotation) was used to identify redundancy or duplication from a set of correlated variables.

3. Results

3.1. Methylmercury exposure and iron status

Overall, about 31.0% of the females examined reported to eat fish daily (95% CI: 25.9–36.8), and the more the geographical isolation, the more frequent the fish intake. Prevalence of daily fish eaters was 6.1% (95% CI: 0.0–15.4) in Area 1, 24.4% (95% CI: 12.3–37.8) in Area 2, 28.6% (95% CI: 19.6–38.0) in Area 3, 32.0% (95% CI: 19.0–45.1) in Area 4 and 54.5% (95% CI: 41.8–67.8) in Area 5 (Resex Cunião, the most isolated) and, correspondingly, respective frequency of families whose fishing has been the main occupation was 6.1%, 11.1%, 19.0%, 27.9% and 29.3%. As consequence, regarding Hair-mercury values, Area 5 was statistically different from Area 1 ($P = .008$), Area 2 ($P = .005$), Area 3 ($P = .003$) and Area 4 ($P = .009$) using Mann–Whitney U test. The median (interquartile range; 95th percentile) values for the Areas 1–5 were, respectively: 3.2 (1.6–5.3; 20.5), 4.2 (1.8–5.8; 11.2), 3.1 (1.6–7.0; 20.6), 2.3 (.8–9.2; 34.7) and 5.5 (3.7–8.0; 13.8). Considering all assessed individuals from the five groups ($N = 274$), the median (interquartile range; 95th percentile) values were 3.8 (1.5–6.6; 17.6). The broad dispersion of Hair-mercury values represents a heterogeneous exposure to MeHg due to wide-ranging fish consumption and differences in species availability. There were no differences among the five assessed areas with respect to age or serum ferritin concentration using Kruskal–Wallis test; $P = .521$ and $P = .442$, respectively.

The main data were grouped according to self-reported weekly frequency of fish meals ($\leqslant 3$ and >3 meals per week) and summarized in Table 1. The groups were similar except by age ($P = .019$). MeHg exposure did not show significant correlation with the two main biomarkers of iron status (serum ferritin and blood hemoglobin) and with serum iron concentration. Hair-mercury and age were weakly correlated ($r = .148$; $P = .014$). Also, Hair-mercury showed a weak positive correlation with MCV ($r = .141$; $P = .020$) and MCH ($r = .132$; $P = .029$).

Most of the females examined between 12 and 49 years showed serum ferritin and blood hemoglobin concentrations (the main parameters in iron status assessment) above the WHO recomendations (WHO, 2001; de Benoist et al., 2008). The estimated prevalence of iron deficiency (ferritin <15 μg L^{-1}) was 16.1% (95% CI: 11.9–20.3) and prevalence of anemia (hemoglobin < 12 mg dL^{-1}) was 28.5% (95% CI: 22.8–33.8). According to hemoglobin limits for nonpregnant females (Rastogi and Mathers, 2000), the estimated prevalence of moderate anemia (hemoglobin <10.9 mg dL^{-1}) in this population was 0.7% (95% CI: 0.0–1.7); there was only one individual who showed severe anemia with hemoglobin concentration of 7.6 mg dL^{-1} (limit for severe anemia = hemoglobin <8 mg dL^{-1}). In summary, serum ferritin, serum iron and blood hemoglobin concentrations were quite homogeneous along the reproductive age, what suggest there is no specific age subgroup at risk within this range. None of the subjects showed exceptional abnormal thyroid function (a potential problem in serum ferritin interpretation). There were only three suspected cases of mild hypothyroidism (TSH >5.6 mcUI mL^{-1}) and one suspected case of subclinical borderline hyperthyroidism (T4 = 1.85 ng dL^{-1}), which were not excluded.

3.2. Linear regression model

When a multiple linear regression analysis was used to assess the relationship between Ln-transformed Hair-mercury (as dependent variable) as a function of age and Ln-transformed serum ferritin concentration, the model showed a weak association: age (as a covariate) had a significant coefficient (Beta = .015; 95% CI: .003–.027; $P = .016$), but Ln serum ferritin did not (Beta = .034; 95% CI: −.147 to .216; $P = .711$). Fig. 2 shows the age-adjusted smoothed plot of Ln Hair-mercury versus Ln serum ferritin.

3.3. Cluster analysis

Hierarchical agglomerative clustering method using average linkage between groups was used to group variables into homogeneous and distinct clusters (rather than observations) with basis on squared Euclidean distance. Since measures of distance depend on the units in which variables are measured and are influenced by whichever variable that takes numerically larger values, the variables were standardized so that they have mean 0 and variance 1 (before applying cluster analysis). Fig. 3 shows the visual representation of the distance at which clusters are combined (dendogram). The observed distances are presented into the range of 1–25, but the ratio of the rescaled distances within the dendrogram is the same as the ratio of the original distances. Ln-transformed Hair-mercury and age presented the smallest similarities in relation to blood hemoglobin concentration, hematocrit, RBC, MCV, MCH, Ln-transformed serum ferritin concentration, MCHC and serum iron concentration (all variables associated to iron status).

Table 1
Iron status assessment of the study population grouped according to self-reported weekly frequency of fish meals and respective bivariate correlation with total-mercury concentration in hair.

	$\leqslant 3$ Times/week $N = 133$	>3 Times/week $N = 136$	P value[a]	Total[b] $N = 274$	r (P value)[c] $N = 274$
Hair-Hg (ppm)	3.9 (1.5–6.0)	3.6 (1.7–6.9)	.549	3.8 (1.6–6.6)	1
Age (years)	24.0 (14.1–35.0)	29.0 (17.8–38.0)	.019*	27.0 (15.1–36.0)	.148 (.014)*
Serum ferritin (mcg L^{-1})	36.1 (23.4–52.8)	38.1 (30.3)	.764	36.2 (22.8–52.9)	.037 (.545)
Blood hemoglobin (g dL^{-1})	12.4 (11.9–13.1)	12.6 (11.7–13.3)	.953	12.6 (11.8–13.2)	.104 (.086)
Hematocrit (%)	37.5 (35.7–40.0)	38.1 (35.4–40.4)	.999	37.6 (35.6–40.2)	.112 (.065)
RBC (10^6 mc L^{-1})	4.5 (4.2–4.7)	4.5 (4.2–4.7)	.155	4.5 (4.2–4.7)	.023 (.707)
MCV (fL)	83.8 (81.3–86.9)	85.4 (81.6–88.4)	.079	84.7 (81.4–88.0)	.141 (.020)*
MCH (pg)	27.9 (26.9–28.9)	28.3 (26.9–29.2)	.164	28.1 (26.9–29.0)	.132 (.029)*
MCHC (g dL^{-1})	33.2 (32.5–33.7)	33.1 (32.5–33.5)	.546	33.2 (32.5–33.6)	–.029 (.638)
Serum iron (mcg dL^{-1})	76.0 (54.0–99.0)	81.0 (54.0–99.5)	.907	78.0 (54.0–99.0)	.049 (.420)

Data expressed as median (interquartile range); ppm = mcg g^{-1}; RBC: Red blood cells count; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentrations.

[a] Mann–Whitney U test.

[b] Including the 5 individuals that did not answer the question about frequency of fish intake.

[c] Pearson's correlation (serum ferritin and hair-Hg were Ln transformed).

* Significant P values ($P < .05$).

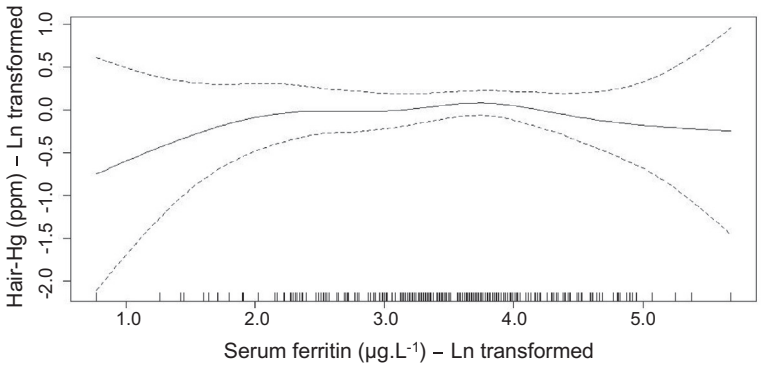

Fig. 2. Age-adjusted plot from a Generalized Additive Model: total mercury concentration in hair (ppm) as a smoothed function of serum ferritin concentration ($\mu g\,L^{-1}$), adjusted for age. Dashed lines: 95% confidence interval; the columns at the *x*-axis represent the distribution of serum ferritin values; $N = 274$.

3.4. Factor analysis

For a factor analysis (Table 2), the variables were initially subjected to a principal component extraction method using ones as prior communality estimates ($N = 274$). Before analysis, each variable was transformed so that it has a mean 0 and a variance 1. Then, principal axis method was used to extract the components, followed by a varimax (orthogonal) rotation with Kaiser Normalization. Combined, the four retained components (eigenvalues >1) accounted for 77.4% of the total variance. Ferritin, the best isolated predictor of iron stores, loaded mainly on the first component (.526) preceded by MCH (.972) and MCV (.900), and followed by MCHC (.503) and hemoglobin (.459). The second component was almost exclusively loaded by hematocrit (.941), CBC (.920) and hemoglobin (.864) whereas serum iron (.867) was the main contributor for the third component, with some minimal contribution of MCHC (.470) and ferritin (.361). Regardless all the variables associated with iron status, Hair-mercury had loads close to zero in the first three component and loaded only the fourth one (.804) together with age (.695).

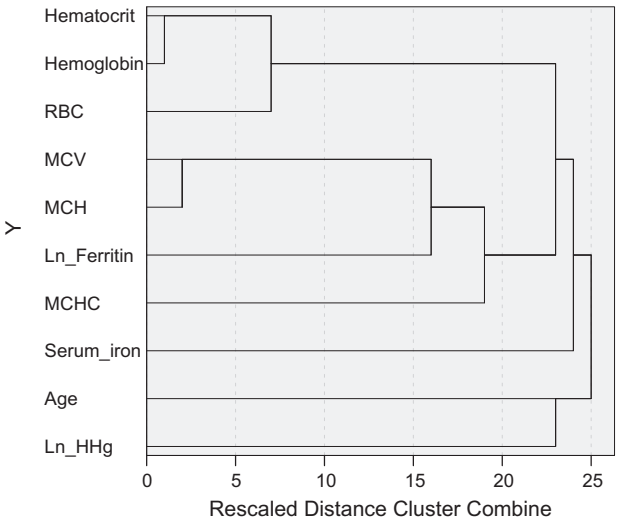

Fig. 3. Dendogram of hierarchical agglomerative clustering analysis using average linkage to group variables with basis on squared Euclidean distance ($N = 274$ individuals). Ferritin and HHg were Ln transformed and all variables were standardized with a mean 0 and variance 1 (before applying cluster analysis). Hemoglobin: blood hemoglobin concentration; RBC: red blood cells count; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; Ferritin: serum ferritin concentration; MCHC: mean corpuscular hemoglobin concentration; HHg: total mercury concentration in hair.

We performed the same analysis after excluding serum iron concentration, since it loaded exclusively on the third component in the first analysis and was not associated with the others. In this second approach, there were three retained components (eigenvalue >1), which accounted for 73.5% of the total variance. The key variables that loaded on the first component were those usually associated with the iron stores: serum ferritin (.595), MCHC (.614), MCV (.835) and MCH (.951), though hemoglobin also had some contribution (.447). The variables that mainly loaded on the second component were those naturally associated with the amount of red blood cells and hemoglobin on blood: blood hemoglobin concentration (.877), RBC (.911) and hematocrit (.950). Hair-mercury (.773) and age (.690) loaded exclusively on the third component.

Finally, in a third similar assessment, two retained components (eigenvalues >1) accounted for 62.7% of the total variance and the interpretation were quite similar when only four primary variables were included: blood hemoglobin concentration, serum ferritin (body iron stores), Hair-mercury (MeHg exposure) and age. Hemoglobin (.828) and serum ferritin (.818) loaded on the first component and their loadings were close to zero in the second component whereas the variables age (.764) and Hair-mercury (.747) loaded on the second component and showed loadings close to zero on the first one.

Regardless the three different ways that variables were included in a principal component analysis using the Kaiser stopping criterion (i.e., all factors with eigenvalues >1), more than one component accounted for a meaningful amount of variance. We therefore retained only these for interpretation. The rotated component pattern obtained in these analyses (particularly in the third approach) seemed to demonstrate simple structure because each component had high loadings for some variables and low loadings for the others. As a varimax rotation is an orthogonal rotation, it results in uncorrelated components. Regarding principal component analysis, Hair-mercury and age loaded on the same component, which had no correlation with the others.

4. Discussion

This cross-sectional study explores relationships between two major predictors concerning cognition and different analyses point to the same important conclusion that iron status is unassociated with Hair-mercury concentration and is therefore not a confounder in studies in which cognitive deficits are correlated to MeHg exposure. In fact, considering different statistical approaches (bivariate correlation, linear regression, hierarchical agglomerative clustering and principal components analysis), there were no findings that suggested a significant association between MeHg exposure and

Table 2
Rotated component matrices of factor analysis containing the loadings of the variables according to three different approaches: all variables included, all variables except serum iron concentration, and only the four primary variables concerning iron status and MeHg exposure.

Components	All variables				Iron excluded			Primary	
	1st	2nd	3rd	4th	1st	2nd	3rd	1st	2nd
Ln HHg	.005	.093	.145	.804	.010	.086	.773	.039	.747
Age	.134	−.080	−.161	.695	.090	−.078	.690	.050	.764
Ln Ferritin	.526	.172	.361	.081	.595	.185	.000	.818	.083
Hemoglobin	.459	.864	.094	.016	.447	.877	.022	.828	.014
Hematocrit	.327	.941	−.030	.037	.286	.950	.071		
Red cells	−.303	.920	.063	−.033	−.301	.911	−.084		
MCV	.900	.183	−.129	.108	.835	.209	.227		
MCH	.972	.047	.040	.089	.951	.074	.159		
MCHC	.503	−.340	.470	−.085	.614	−.327	−.189		
Serum iron	−.022	.086	.867	−.012					

Extraction method: principal component analysis; Rotation method: varimax (uncorrelated components); Kaiser stopping criterion retained only components with eigenvalues >1. Ferritin and HHg were Ln transformed. $N = 274$ individuals. Loadings <.350 showed in grey. HHg: total mercury concentration in hair; Ferritin: serum ferritin concentrataion; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

iron status in riparian females of bearing age. Despite with the focus on a riparian Amazonian population, these results strengthen the global discussion on which (and how strongly) nutrients present in fish and maternal diets are confounders in detecting associations between prenatal fish-mercury exposure and child neurodevelopment, which has important public health implications (Davidson et al., 2008).

The median Hair-mercury values in this sample (3.8 ppm) were relatively low, if compared with previous results concerning females in fertile age from Madeira River. This fact may be a natural consequence of changes in the pattern of fish consumption and, also, in the availability of the species since the regional development gives people more options of food. In 1991, for example, (Boischio and Barbosa, 1993) assessed communities downstream Santo Antônio rapids (including Resex Cuniã) and found approximately more than twice the median value found in our study – 37 from a total of 70 females of childbearing age (53%) had Hair-mercury >10 ppm. In this region, similar to other parts of Amazon, fish consumption tended to be higher among the more isolated and impoverished communities (Fonseca, 2008; Monrroy et al., 2008; Dutra et al., 2012), though opposite conclusions have been found, for example, in the United States (U.S. Environmental Protection Agency, 2013).

Iron deficiency (the primary cause of anemia) has been one of the most severe and important nutritional deficiencies in the world and women of childbearing age are one of the most vulnerable groups (Murray-Kolb, 2011). In the last decade, the prevalence of anemia in young females (15–59 years of age) was 42.3% in nonindustrialized countries (WHO, 2001) and 23.1% (95% CI: 8.4–49.4) in Brazil (de Benoist et al., 2008). About 9.2–15.7% of nonpregnant young females (12–49 years of age) have been affected by iron deficiency in United States (Cogswell et al., 2009). Although there are many causes of anemia besides iron deficiency, about 2–5 times more iron-deficient than iron-deficient-anemic individuals in tropical regions are expected according to World Health Organization (WHO, 2001). Among possible explanations for the estimated prevalence of anemia (28.5%) that was higher than the prevalence of iron deficiency (16.1%) in this study, the presence of inflammatory conditions that increase ferritin values may be a point of concern.

Serum ferritin concentration has been the most specific biochemical correlate of iron stores and was used to adjust iron dosage in individual iron prophylaxis both before and in early pregnancy (Milman et al., 2006). However, serum ferritin increases in inflammatory and infectious conditions, as do many other acute-phase proteins, including CRP. Actually, the acute phase response may be triggered with CRP values that are considerably lower than 6 mg L^{-1} (Abraham et al., 2003), the detection limit used in this study. Some authors have suggested to raise the cutoff for the definition of a low ferritin concentration (i.e. from 15 to 30 or 50 μg L^{-1}) to account for the influence of inflammation on ferritin when concerning public health policies in infected populations (Beard et al., 2006). In this case, for example, if we had used 30 μg L^{-1} as a cut-off for serum ferritin concentration, estimated prevalence of iron deficiency had increased to 40.1% (95% CI: 34.0–45.7).

Additionally, possible causes of high serum ferritin without iron overload include liver disease (including hepatitis and steatosis), chronic inflammatory conditions (i.e. rheumatoid arthritis and inflammatory bowel disease), malignancies (especially hematological), subclinical bacterial infections, familial hyperferritinemia and cataract syndrome, alcoholism and thyrotoxicosis. In this study, alcohol excess and thyrotoxicosis were appropriately assessed (no case was identified), and the three self-reported cases of rheumatoid arthritis were excluded.

After the onset of the inflammation, the serum iron concentration falls rapidly as part of the acute phase response irrespective of the status of the iron stores in the body (condition not verified in this study). In fact, overall, most young females had serum iron concentration >50 μg dL^{-1} that may reflect a regular exposure to iron. The significant contribution of contaminant iron (predominantly in the non-haem form, which is poorly absorbed) to total iron intake has been well documented in the diets of many populations in developing countries where food with iron from soil, dust and water and the practice of geophagy are common (Harvey et al., 2000). Indeed, since both deficiency or excess can affect many enzymatic and structural proteins, iron metabolism is one of the most tightly regulated events and its absorption is favored when iron stores is low (Youdim, 2008).

Similarly to other Amazonian studies, there was no adjustment for seasonal variation in hair sampling since realistic aspects of field activities did not permit that hair samples were collected at the same time. Therefore, results do not include some seasonal variation in methylmercury exposure, which may exist along the year. In this approach, seasonality was not controlled because population did not agree with hair sampling twice a year. Another limitation in our study (also usual in Amazonian studies) includes the lack of an accurate quantification of fish intake. Unfortunately, regarding the distance of the houses (sometimes longer than 10 km one from another) and the number of assessed individuals (in all, $N > 2000$ adults and children), it was not possible to quantify the individual consumption in each meal, which will be carried out in sequence. Anyway, seasonal variability in fish availability had little impact on overall Hair-mercury concentrations

throughout the year in the isolated riparian community of Puruzinho Lake in Madeira River Basin (Oliveira et al., 2010).

Methylmercury is neurotoxic, but there are many risk conditions in developing countries and the contribution of mercury exposure may not be clear, given all the noise from other factors, which may be difficult to adjust for (Wasserman et al., 2008). In addition to noise from many covariates, there is also a concern about the precision of the exposure assessment (Canuel et al., 2006; Grandjean and Budtz-Jørgensen, 2010; Karagas et al., 2012). As previously exposed, despite usually chosen, both iron status and MeHg exposures have been assessed through proxy variables, which are far from totally reliable. Objectively: the greater the imprecision of the estimates, the larger the noise from covariates.

Children with deficient neurodevelopment during early life are at risk for later neuropsychological problems, poor school achievement, low-skilled employment, and poor care of their own children, thus contributing to the intergenerational transmission of poverty (Reynolds et al., 2001). Concerning non-genetic factors, the balance of intrauterine exposure to toxicants and protective substances significantly affects the offspring's brain development. This study showed that iron stores have not been a confounder, thus simplifying future studies. Nonetheless, inadequate nutrient supplies, exposures to other neurotoxicants (such as pesticides, cyanide, thimerosal), differences in stimulation, geographical isolation with limited access to technologies, etc. will remain and will cause residual confounding, especially in cross-sectional studies. In the Amazonian riparian communities, where iron deficiency could be a possible confounder, studies concerning cognition would require general nutritional status assessment due to the impact on brain development caused by undernutrition. Furthermore, a demonstration that adjusting for indicators of maternal iron status does not have an appreciable impact on the association between Hair-mercury and some health outcomes may be necessary. Yet, considering fish intake as a negative confounder, a substantial imprecision of its quantification can cause a further underestimation of the mercury effect even after confounder adjustment (Choi et al., 2008).

We conclude that iron status and methymercury exposure probably occur in an independent way in riparian Amazonians.

List of author's contribution to the work

Marlon F. Fonseca. Project planning, participation of field work and writing the manuscript.

Sandra S. Hacon. Coordination of Human Health INOVA ENSP Fiocruz Program; project planning, organization and participation of all field work that included assessment of human beings.

Philippe Grandjean. Supervision of the manuscript preparation; discussion about the main points concerning mercury toxicology, human reproduction and interaction among predictors.

Anna L. Choi. Revision of manuscript; supervision of statistical analysis.

Wanderley R. Bastos. Coordination of the Project with focus on environmental aspects, including chemical analysis for determination of mercury concentration in biological samples.

Acknowledgements

We sincerely thank the Madeira Riverine communities, the professionals from the Health and Education Municipal Secretariat (including the teachers and directors of the state and municipal primary schools), the nurses Nubia Gomes, Andreia Vasconcelos and Janaina Mara for field support, Gabriela Protazio da Silva for mercury analysis, Prof David Bellinger and Prof Olaf Malm for reviewing the drafts and Prof Reinaldo Calixto (in memoriam) and Rodrigo Gonçalves for technical support in chemical analysis.

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