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Novel biomarkers of mercury-induced autoimmune dysfunction: a Cross-sectional study in Amazonian Brazil

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Abstract

Mercury is an ubiquitous environmental contaminant, causing both neurotoxicity and immunotoxicity. Given its ability to amalgamate gold, mercury is frequently used in small-scale artisanal gold mining. We have previously reported that elevated serum titers of antinuclear autoantibodies (ANA) are associated with mercury exposures of miners in gold mining. The goal of this project was to identify novel serum biomarkers of mercury-induced immunotoxicity and autoimmune dysregulation.

We conducted an analysis of serum samples from a cross-sectional epidemiological study on miners working in Amazonian Brazil. In proteomic screening analyses, samples were stratified based on mercury concentrations and ANA titer and a subset of serum samples (N=12) were profiled using Immune Response Biomarker Profiling ProtoArray protein microarray for elevated autoantibodies. Of the up-regulated autoantibodies in the mercury-exposed cohort, potential target autoantibodies were selected based on relevance to pro-inflammatory and macrophage activation pathways. ELISAs were developed to test the entire sample cohort (N=371) for serum titers to the highest of these autoantibodies (anti-glutathione S-transferase alpha, GSTA1) identified in the high mercury/high ANA group.

We found positive associations between elevated mercury exposure and up-regulated serum titers of 3760 autoantibodies as identified by ProtoArray. Autoantibodies identified as potential novel biomarkers of mercury-induced immunotoxicity include antibodies to the following proteins: GSTA1, tumor necrosis factor ligand superfamily member 13, linker for activation of T cells, signal peptide peptidase like 2B, stimulated by retinoic acid 13, and interferon induced

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transmembrane protein. ELISA analyses confirmed that mercury-exposed gold miners had significantly higher serum titers of anti-GSTA1 autoantibody [unadjusted odds ratio = 89.6; 95% confidence interval: 27.2, 294.6] compared to emerald miners (referent population).

Mercury exposure was associated with increased titers of several autoantibodies in serum including anti-GSTA1. These proteins play a wide variety of roles, including as antioxidants, in the regulation of pro- and anti-inflammatory cytokines, as well as danger and oxidative stress signaling. Dysregulation of these proteins and pathways is believed to play a role in autoimmune diseases such as rheumatoid arthritis, Sjögren's syndrome, and multiple sclerosis. Taken together, these results suggest that mercury exposure can induce complex autoimmune dysfunction and the immunotoxic effects of this dysfunction may be measured by serum titers to autoantibodies such as anti-GSTA1.

1. Introduction

Mercury is a naturally occurring element and ubiquitous environmental contaminant released from combustion of coal and fossil fuels; mining operations; and metal, cement, and chlor-alkali production (WHO 2007, 2010). Elemental mercury is the primary form of mercury found in the atmosphere where it is stable for approximately 2 years and travels vast distances around the globe (Muir et al. 2009; Nguyen et al. 2010). Elemental mercury can be oxidized in the atmosphere to inorganic mercury which then is returned to the ground by dry and wet deposition. Inorganic mercury contaminates waterways, can be biotransformed to methylmercury, and bioaccumulate in piscivorous species of fish. Consumption of methylmercury-laden fish represents the most common route of exposure for humans (National Research Council (US) Committee on the Toxicological Effects of Methylmercury 2000).

Mercury has been shown to cause damage and dysfunction in a number of physiological systems and has been well-documented as detrimental to the neurodevelopment of infants and children (National Research Council (US) Committee on the Toxicological Effects of Methylmercury 2000; WHO 2010). All mercurial species are toxic, differing in toxicodynamics, toxicokinetics and toxicological effects partly due to differences in solubility and bioavailability (Clarkson 1997; Gardner et al. 2010a; National Research Council (US) Committee on the Toxicological Effects of Methylmercury 2000; WHO 2010).

A more recent area of research focus has been on the immunotoxic properties of mercury compounds. Dysregulation in the pro- and anti-inflammatory cytokine balance as a result of mercury exposure has been documented (de Vos et al. 2007; Gardner et al. 2009; Gardner et al. 2010b; Hemdan et al. 2007). In a recent study by Gardner *et al* (2009) human peripheral blood mononuclear cells (PBMCs) were exposed to inorganic mercury at physiologically relevant concentrations. Only lipopolysaccharide-stimulated PBMCs responded to mercury *in vitro* and produced a concentration-dependent increase in release of pro-inflammatory cytokines interleukin (IL)-1 β and tumor necrosis factor- α with a concurrent decrease in anti-inflammatory cytokines IL-1Ra and IL-10. In the same *in vitro* system, methylmercury exposure caused similar cytokine modulation (Gardner et al. 2010a). Interestingly, opposite

effects on cytokine production were observed in response to methylmercury when PBMCs were stimulated with monoclonal antibodies against T-cell receptors (Hemdan et al. 2007) or with T-cell mitogen concanavalin A (de Vos et al. 2007). These findings suggest differential effects of mercury compounds upon immune cell subsets.

In a cross-sectional study of populations exposed to methylmercury and inorganic mercury as a result of small-scale artisanal gold mining in the Brazilian Amazon, we previously demonstrated that mercury exposure (both inorganic mercury and methylmercury) was positively correlated with elevated serum titers of antinuclear autoantibodies (ANA) (Gardner et al. 2010b; Nyland et al. 2011a; Silva et al. 2004). When the mining population was dichotomized based on mercury exposure and ANA positivity, we found that the high mercury/high ANA group had significantly elevated serum concentrations of pro-inflammatory cytokines IL-1 β , tumor necrosis factor- α , and interferon- γ compared to those with low mercury exposure (Gardner et al. 2010b).

These studies provide evidence that mercury exposure may induce autoimmune dysfunction and systemic inflammation in affected populations. However, these analyses were relatively limited to analysis of two biomarkers of autoimmune dysfunction. Further, in our study of maternal-cord blood pair samples, we did not find a correlation between methylmercury exposure and ANA (Nyland et al. 2011b). Thus we may not have identified consistent biomarkers of mercury-induced immune dysregulation. Such information would benefit studies in populations exposed to low levels of mercury and provide a potential early indicator of mercury-induced exacerbation of autoimmunity (although not autoimmune disease). The goal of this research project was to extend the analysis of autoantibodies in serum samples from persons exposed to mercury in mining populations from the Brazilian Amazon.

2. Materials and Methods

2.1. Study population

This study utilized serum samples collected from participants in a larger epidemiological study conducted in collaboration with the Fundação Nacional de Saúde (FUNASA) of the Brazilian Ministério da Saúde in the states of Pará and Goiás as described previously (Gardner et al. 2010b). After obtaining informed consent, subject enrollment and sample collection were completed as previously described (Gardner et al. 2010b; Silva et al. 2004). This study was approved and supervised by the institutional review boards of the University of South Carolina and FUNASA, as well as the Committee on Human Research at the Johns Hopkins Bloomberg School of Public Health. Briefly, at each recruitment site, enrollment and sampling locations were established in central public locations. At the emerald and diamond mining sites, study enrollment occurred for one day and covered multiple shifts of workers. All miners at each study site were invited to participate, and we estimate that 100% of the population present on the enrollment/sampling day was enrolled in the study. At the gold-mining site, study enrollment occurred for five days. All residents of the camp were invited to participate in the study. For this population, it is difficult to estimate the percent enrollment because of the dispersion of the gold miners at distant locations from the camp; however, 100% of those in the gold-mining camp who were asked to participate did so. For

all sites, the only exclusion criterion was self-disclosed indication that the individual was not involved in mining.

For this study, we utilized two groups, one at a gold-mining camp and one at two other mining sites. At the gold-mining camp, Rio-Rato in the state of Pará, the population was directly involved in small-scale artisanal gold mining operations, resulting in high exposures to elemental mercury as described elsewhere (Crompton et al. 2002; Silva et al. 2004). For the referent population, we utilized samples from diamond miners from regions of Davinópolis and São Antônia Rio Verde and emerald miners from Campo Verde-Itaobi and Campo Verde-Vereador, all located in the state of Goiás.

Serum samples were frozen on site and stored at the Instituto Evandro Chagas in Belem Brazil. An aliquot was taken from these stored samples prior to shipment to the United States where it was stored at -80°C until use in our previous studies (Gardner et al. 2010b; Silva et al. 2004), as well as this study.

2.2. Mercury exposure

Mercury exposure was assessed by occupational history and past exposure information collected by questionnaire as well as biomarker sampling. Mercury exposure for Rio-Rato was determined by urine analysis, while for the diamond and emerald miners, mercury exposure was determined by hair analysis. Unfortunately hair was not collected for the Rio-Rato population. Different measures were utilized in accord with standard practice in which urine is the preferred compartment for assessing ongoing occupational exposures to elemental and inorganic mercury and hair is the preferred compartment for assessing nonoccupational exposures to methylmercury (usually associated with contaminated fish consumption) (Clarkson 1997). Cold vapor atomic absorption spectrophotometry standard methods were used to measure mercury in both biologic matrices. Mercury analyses were conducted by a laboratory of FUNASA that participates in an international QA/QC program with the Université du Québec à Montréal. Mercury levels in these populations have been previously described (Gardner et al. 2010b; Silva et al. 2004).

2.3. Screening ProtoArray analyses

2.3.1. Sample selection—For the specific autoantibody analyses (see section 2.4) we utilized the entire sample cohort described in (Table 1). As with gene expression arrays, use of protein arrays as a screening tool is a standard protocol for generating hits that can then be explored in the whole population. For the screening ProtoArray analyses, a small subgroup of samples was selected using the strategy employed in our studies of cytokines in this same population (Gardner et al. 2010b); that is, stratified on the basis of mercury biomarker levels and ANA titer. These samples were categorized into (1) high mercury [N=6] or (2) low mercury [N=6]. The high mercury samples were randomly selected from the gold miners based on elevated urinary mercury concentrations. A cut-off of $3.0\text{ }\mu\text{g/L}$ urine, which represents the 95th percentile for adults within the US, based on the geometric mean and standard deviation reported by Dye *et al* (2005). The low mercury samples were randomly selected from the diamond and emerald miners based on their lower hair mercury levels

(Gardner et al. 2010b). A cut off of 1.73 µg mercury/g hair was used based on the 95th percentile reported by McDowell *et al* (2004) for hair mercury levels in US adults.

Within these two categories of mercury exposure, we further stratified the sample based on their ANA titers into (1) high mercury/low ANA [N=3], (2) high mercury/high ANA [N=3], (3) low mercury/low ANA [N=3], or (4) low mercury/high ANA [N=3]. Participants with no detectable ANA in their serum, as evaluated at a 1:10 dilution by indirect immunofluorescence method on HEp-2 slides (INOVA Diagnostics) as in Burek and Rose (1995), were chosen for the low ANA category. Participants with ANA titers greater than 1:80 and ranging to greater than 1:320, as determined by the highest dilution at which immunofluorescence was detectable, were chosen for the high ANA category. This division of cohorts was utilized in our previous studies in this (Gardner et al. 2010b) and other cohorts (Nyland et al. 2011a). It should be noted that participants from the gold mining and diamond mining populations had more reports of prevalent malaria compared to residents of the emerald mining community (Gardner et al. 2010b). All samples selected for preliminary protein array analyses were from adult females.

2.3.2. ProtoArray analysis—An aliquot of each selected serum sample (N=12, selection criteria described above) was analyzed with a human ProtoArray platform optimized for immune response biomarker profiling containing 9,400 immobilized non-redundant native human proteins expressed in a baculovirus system, purified from insect cells, and printed in duplicate onto a nitrocellulose-coated glass slide (ProtoArray Human Microarray v5.0 for IRBP, Invitrogen), according to the manufacturer's protocol. ProtoArrays contained protein kinases, transcription factors, membrane proteins, nuclear proteins, signal transduction proteins, secreted proteins, cell communication proteins, proteins involved in metabolism, and cell death proteins. In brief, the arrays were blocked with synthetic block (Invitrogen), probed with sample serum (1:500 dilution), and autoantibodies detected using Alexa Flour 647-conjugated goat anti-human IgG. Arrays were scanned with ScanArray Express HT scanner (Perkin Elmer). Array images with associated fluorescence intensity were mined for analysis of the data with Protoarray Prospector (Invitrogen, v5.2.1) software under the immune response profiling mode. All proteins with a z-factor<0.4 compared to internal array controls and a signal intensity greater than 5,000 were included in the differential expression analysis presented in Venn diagrams (Figures 1 and 2), constructed using Venn Diagram Plotter (v1.4.3740, Pacific Northwest National Laboratory) for two groups (proportional) or Microsoft Photoshop for four groups (non-proportional). Any differences in intensities between exposure groups with a p-value< 0.05 were considered significant and included in further *in silico* pathway analysis (Ingenuity Pathway Analysis, Ingenuity Systems). Multiple analyses were conducted for the selection of novel biomarkers. Pathways of interest were those involved in antigen presentation, oxidative stress and macrophage signaling/activation, and immunologic pathways relevant to previous mercury immunotoxicology research.

2.3.3. Generation of ProtoArray heat map—The heat map (a graphical representation of data wherein individual values along a continuum are represented as colors) was created by setting a baseline level to autoantibody signal intensities centered about the median level

of the autoantibody intensities. The city block method (a hierarchical cluster analysis to evaluate distances along two dimensions wherein the sum of the two dimensional distances is used) of similarity test was employed for hierarchical clustering using the freeware programs Cluster 3.0 (Eisen Lab, v2.11) and Treeview (Eisen Lab, v2.11) (Eisen et al. 1998).

2.4. Anti-glutathione S-transferase alpha enzyme-linked immunosorbent assay

Of the potential novel biomarkers identified from the screening ProtoArray analysis, we selected one for further validation in the full data cohort using a custom enzyme-linked immunosorbent assay. We chose the autoantibody with the highest average titer that was unique to the high mercury/high ANA samples, GSTA1. GSTA1 was also selected because it is one of a family of xenobiotic detoxification enzymes whose expression may be impacted by mercury exposure. Thus, the development of antibodies against this protein may indicate a mercury-specific host-response.

2.4.1. Synthetic peptides and anti-serum—Descriptions of the synthetic human immunodominant peptides corresponding to immunodominant epitopes for the protein GSTA1 are summarized in Table 1 and were purchased from Primm BioTech (Cambridge, MA). The peptides were selected on a ranking basis for immune-dominance for presentation by antigen presenting cells selected using proprietary software developed by Primm BioTech. Anti-GSTA1 anti-serum was generated according to standard protocols in three rabbits by immunization with a cocktail of the three peptides in Table 2 by Primm BioTech. The positive anti-serum was affinity purified prior to use. Pre-immune serum served as negative control.

2.4.2. Enzyme-linked immunosorbent assay analysis—The full sample cohort (N=371) of serum samples from the gold, diamond, and emerald mining populations was utilized for enzyme-linked immunosorbent assay to validate the protein array data. Immulon2 plates (ThermoScientific) were coated with peptide cocktail including all three peptides (Table 1) (0.25 µg/ml in PBS) and incubated at 4°C overnight. The plates were washed with wash buffer (2% FBS in PBS) and diluted serum samples (1:500 and 1:2500 in PBS) were added in triplicate. A triplicate 8-point standard curve was generated with 2-fold serial dilutions of rabbit anti-serum and negative controls included two dilutions of pre-immune serum (1:500 and 1:2500). Plates were incubated for 2 hours at room temperature, washed, and peroxidase-conjugated recombinant protein G (Thermo Scientific) (0.1 µg/ml) was added. Anti-GSTA1 relative titer was determined by colorimetric analysis with TMB substrate solution (Sigma) at 450nm. The limit of detection was set per plate as the standard deviation of the blanks × 3. Any reading below the limit of detection was set to the limit of detection/ 2.

2.5. Statistical analysis

Given the skewed distribution of mercury measurements and serum autoantibody titers, these biomarker levels were log-transformed for analysis. For plotting, the median and inter-quartile ranges are given for each measurement on their natural scale. Log-transformed anti-GSTA1 values were compared using one-way analysis of variance (ANOVA) followed by

pairwise post-tests using the Bonferroni correction. We used simple logistic regression to model the correlation between anti-GSTA1 titer and log-transformed mercury exposure among individual (i) diamond miners (DM) or gold miners (GM) compared to emerald miners (EM). Here Y_i represents the anti-GSTA1 status of the i th individual: ***Model 1

$$\begin{aligned} Y_i &\sim \text{Bernoulli}(p_i) \\ \log \text{it}(p_i) &= \beta_0 + \beta_1(DM_i) + \beta_2(GM_i) \end{aligned} \quad \text{Model 1}$$

Although diamond and emerald miners could both be considered appropriate referent groups since they are so similar in occupational and environmental exposure conditions, emerald miners were chosen as the referent group as previously (Gardner et al. 2010b). Because sex and malarial status are potential confounders and could potentially influence autoantibody titer, these were also tested in a simple logistic regression model: Model 2

$$\begin{aligned} Y_i &\sim \text{Bernoulli}(p_i) \\ \log \text{it}(p_i) &= \beta_0 + \beta_1(DM_i) + \beta_2(GM_i) + \beta_3(\text{sex}_i) + \beta_4(\text{malarial status}_i) \end{aligned} \quad \text{Model 2}$$

All statistical analyses were conducted in and data graphed using STATA (Stata Corp, v10.IC).

3. Results

3.1. Mercury exposure increases autoantibody levels

Population characteristics for each recruiting location (community) within each mining population are summarized in Table 1. In general the majority of the participants were males. As expected, a higher proportion of the river mining populations, gold and diamond miners, had prevalent malaria at the time of recruitment into the study compared to emerald miners. This is similar to our previous report on these populations (Gardner et al. 2010b), although the number of participants included in this study for the gold mining population (Rio-Rato) is 223 rather than only 98.

Screening proteomic analyses showed that mercury exposure significantly increased serum titers to self-antigens since those samples from the high mercury group had elevated serum titers (with a signal of greater than 5,000 on the ProtoArray compared to internal controls) to a total of 3,760 unique proteins of the 9,400 proteins examined compared to the low mercury group with 49 unique proteins (Figure 1). Further, the high mercury/high ANA group had elevated serum titers to 1383 unique proteins compared to the low mercury/low ANA, low mercury/high ANA, and high mercury/low ANA groups with elevated titers to 22, 27, and 407 unique proteins, respectively (Figure 2, Table 3).

Of the 1383 unique autoantibodies identified in the high mercury/high ANA group by ProtoArray analysis, we selected proteins with the highest serum autoantibody titers and at least 10-fold increased levels compared to the low mercury/low ANA group from across all cellular compartments for further analysis. Rather than simply selecting the autoantibodies with the highest level, by selecting the highest across the cellular compartments, we were better able to examine the diversity of mercury-associated effects. Individual protein array

normalized intensities for each sample (three per group based on mercury and ANA status) for this subset of selected self-antigens is shown in Figure 3. We examined one nuclear protein: stimulated by retinoic acid 13 and three transmembrane proteins: linker for activation of T cells, signal peptide peptidase like 2B, and interferon induced transmembrane protein. Cytosolic proteins examined included GSTA1 and tumor necrosis factor ligand superfamily member 13. Average serum autoantibody titers against all of these self-proteins were upregulated more than 10-fold in the high mercury/high ANA samples compared to low mercury/low ANA samples (Figure 3).

3.2. Mercury exposure significantly increases anti-glutathione S-transferase alpha autoantibodies

We chose to focus further analysis on anti-GSTA1 autoantibodies that was the most highly upregulated in the high mercury/high ANA group. GSTA1 is one of a family of detoxification enzymes responsible, in part, for metabolism of byproducts of oxidative stress (Hayes et al. 2005; Hayes and McLellan 1999). As shown in Table 4, gold miners had significantly higher serum titers of anti-GSTA1 autoantibodies as compared to diamond and emerald miners ($p < 0.001$). While mercury measures were continuous variables, there is not yet consensus in the mercury field for a conversion factor to transform urine mercury levels to hair mercury levels. Therefore, we conducted these analyses using mercury exposure as a categorical variable. Using simple logistic regression analysis, gold miners had significantly higher odds of elevated serum anti-GSTA1 autoantibody titers [unadjusted odds ratio = 89.6; 95% confidence interval: 27.2, 294.6] compared to the referent population (Table 5). The significance of this relationship did not change when adjusted for sex and malarial status, both potential confounders in the analysis.

4. Discussion

In this study we expanded our analysis of serum biomarkers of autoimmune dysfunction in mining populations from Amazonian Brazil with and without exposures to mercury. We found that the higher mercury-exposed gold miners had, in total, a higher number of different autoantibodies as compared to the diamond and emerald miners. These autoantibodies were reactive with self-proteins from many different cellular compartments, suggesting that the observed autoimmunity is not driven by a particular sensitivity, but rather as a result of mercury effects on the immune system generally.

Toxicity of metals is often associated with binding to thiol groups on proteins and enzymes, causing loss of function and various adverse health effects (Clarkson 1997; National Research Council (US) Committee on the Toxicological Effects of Methylmercury 2000; WHO 2010). Methylmercury, in particular, is highly mobile within the human body as a result of formation of methylmercury-thiol complexes that are easily transported across membranes (Clarkson 1997; Rubino et al. 2006; Rubino et al. 2004). Cellular entry is hypothesized to be primarily facilitated by complexes with cysteine and homocysteine while exit is via glutathione complexes (Clarkson 1997).

We chose to focus on autoantibodies to GSTA1 for many reasons. In the small subgroup of serum samples tested with screening ProtoArray, the protein with the highest average

autoantibody response in the high mercury/high ANA group was GSTA1. Additionally, the protein itself is of interest given what we know about xenobiotic biology. Glutathione transferases are detoxification enzymes of three families, divided based on their cellular expression: cytosolic, mitochondrial, and microsomal (Hayes et al. 2005). GSTA is one of the glutathione transferases localized to the cytosolic compartment, the largest family of these enzymes, and some studies have shown that GSTA1 has a tendency to associate with cellular membranes (Prabhu et al. 2004). This membrane propensity may impact the accessibility of the protein to the immune system and potentially increase the likelihood for development of anti-GSTA1 antibodies.

Upregulation of glutathione transferase expression levels appears to be a conserved cellular response to oxidative stress (Hayes et al. 2005). Previous studies have examined expression levels of GSTA in liver or plasma (Mulder et al. 1999) and demonstrated variability between males and females. Others have shown that polymorphisms in GSTA1 genes confer protection against immune activation by exposure to xenobiotics in vulcanization processes (Jonsson et al. 2008), presumable because these polymorphisms reduce the enzyme inhibition induced by xenobiotic compound binding to the enzyme (El-Demerdash 2001). While heavy metal exposure and binding may decrease glutathione transferase (but not GSTA) enzymatic activity, exposure to mercury has been shown to increase expression of glutathione transferase mRNAs (Cambier et al. 2012; Korashy and El-Kadi 2006) in cell culture and zebrafish model systems. However, these studies examined expression of the gene transcript or the protein itself rather than the development of autoantibodies reactive with the protein as we have reported here. That is, we have reported on the generation of an autoimmune response to GSTA (serum titers of anti-GSTA1) and not direct measures of protein or transcript levels, providing another measure of mercury-induced autoimmune dysfunction.

GSTA has been shown to play a key role in protection against oxidative stress (Hayes et al. 2005; Liang et al. 2005) and generation of reactive oxygen species is believed to be important in the pathogenesis of certain autoimmune diseases. For example, inflammatory bowel disease and Crohn's Disease both have increased reactive oxygen species levels in the gastrointestinal tract associated with increased inflammation and disease severity (Kruidenier et al. 2003; Kruidenier and Verspaget 2002). Thus, a role for GSTA in protection against xenobiotic-induced exacerbation of autoimmunity and autoimmune disease is appropriate.

4.1. Study strengths

Our previous studies have demonstrated that elevated mercury exposure is associated with ANA serum titers for individuals exposed to either inorganic mercury through small-scale gold mining operations (Gardner et al. 2010b; Silva et al. 2004) or methylmercury through contaminated fish consumption (Nyland et al. 2011a). While ANA is one of the biomarkers used to diagnose systemic lupus erythematosus among other autoimmune diseases (Tan et al. 1982), we have proposed that in our studies of populations exposed to mercury, that ANA is more appropriate as a general indicator of increased autoimmune dysfunction. In

this study, we have identified a novel biomarker, anti-GSTA1 autoantibody, as a new indicator of mercury-induced autoimmune dysfunction.

4.2. Study limitations

The lack of a consistent measure of mercury exposure among the populations described is a major limitation of this study. Unfortunately the same sample matrix was not available for both populations. As stated in the methods section, urine is the appropriate biomatrix for assessing occupational exposures to elemental and inorganic mercury while hair (and blood) is the appropriate biomatrix for examining nonoccupational exposures to methylmercury. Therefore, for these two different mining populations and the expected exposure type, the preferred biological compartment was tested. We controlled for this difference as best we could in the analysis of the data.

While elevated serum titers of anti-GSTA1 autoantibody may be indicative of autoimmune dysfunction, we do not yet have any information of relationship with disease. This is particularly true for this study as health information beyond malarial status in this population is limited. Additionally, the proteomic data are a very rich dataset and need to be further mined for additional relationships to mercury exposure. These additional analyses are ongoing.

4.3. Conclusions

Although this study involved mining populations from Amazonian Brazil who are exposed to inorganic mercury, these results may have relevance for individuals exposed to methylmercury through contaminated fish consumption. Future studies in other mercury-exposed populations will test this hypothesis and the potential for broadened applicability to other populations who consume large amounts of fish from contaminated watersheds.

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Highlights

- We examined levels of autoantibodies in Hg-exposed miners from Amazonian Brazil.
- We found associations between Hg and up-regulated titers of 3760 autoantibodies.
- Enzyme-linked immunosorbent assay confirmed that Hg-exposed gold miners had higher titers of anti-glutathione S-transferase alpha.
- Hg exposure can induce complex autoimmune dysfunction.

Figure 1. Differential autoantibody expression in mercury-exposed populations

Proportional Venn diagram illustrating distribution of up-regulated autoantibody serum titers (as measured by signal intensity greater than 5,000 on Immune Response Biomarker Profiling ProtoArray) as compared to internal array controls for high versus low mercury-exposed individuals (N=6 each). Overlapping area corresponds to the shared autoantibody positivity between the groups.

Figure 2. Differential autoantibody expression in mercury/ANA subgroups

Non-proportional Venn diagram illustrating distribution of up-regulated autoantibody serum titers (as measured by signal intensity greater than 5,000 on Immune Response Biomarker Profiling ProtoArray) as compared to internal array controls for high mercury/high ANA, high mercury/low ANA, low mercury/low ANA, and low mercury/high ANA groups (N=3 each). Overlapping areas correspond to the shared autoantibody positivity between groups. This is a symbolic representation of the relationships between the four groups, but these relationships are not drawn to scale. Relative proportions of the relationships between four groups are not possible in a Venn diagram (Phillips 2014).

Figure 3. Heat map of differential autoantibody levels

The baseline level to data centered about the median level of autoantibody intensities are shown for each sample within each subgroup dichotomized based on differential mercury level and ANA positivity. Fold increase in serum autoantibody levels are shown for +/-+ compared to -/- average intensity for each self-protein.

Table 1

Population characteristics by recruitment site.

Type mining	of Population	N	Mercury (median, IQR)	Sex	Prevalent malaria
Diamond	Davinópolis	n=24	0.48 µg/g hair (0.29-0.63)	male=20 (83%) female=4 (17%)	yes=8 (33%) no=16 (67%)
	Sao Antonio Rio Verde	n=33	0.96 µg/g hair (0.6-1.29)	male=31 (94%) female=2 (6%)	yes=13 (40%) no=20 (60%)
Emerald	Campo Verde, Vereador	n=22	0.268 µg/g hair (0.217-0.398)	male=22 (100%) female=0 (0%)	yes=1 (5%) no=22 (95%)
	Campo Verde, Itaobi	n=69	0.288 µg/g hair (0.222-0.466)	male=35 (51%) female=34 (49%)	yes=1 (1%) no=68 (99%)
Gold	Rio-Rato	n=223	2.6 µg/L urine (0.02-6.43)	male=149 (67%) female=74 (33%)	yes=164 (54%) no=59 (46%)

IQR: inter-quartile range.

Table 2

Human glutathione S-transferase alpha peptides utilized.

	Amino Acid Sequence	Amino Acid Length	Position
Peptide 1	AQSVYAFSARPLAGGEPC	18	13-29
Peptide 2	AGAHPLFAFLREALPC	16	120-134
Peptide 3	CGPDGVPLRRYSRRF	15	170-183

Table 3

Differential autoantibody expression in mercury/ANA subgroups (companion data for Figure 2).

	low Hg/low ANA	low Hg/high ANA	high Hg/low ANA	high Hg/high ANA
low Hg/low ANA	3284			
low Hg/high ANA	161 (0)	3587		
high Hg/low ANA	313 (6)	305 (4)	8027	
high Hg/high ANA	337 (25)	335 (29)	2422 (1970)	8912

Shaded cells present the total number, for each subgroup, of up-regulated autoantibodies (as measured by signal intensity greater than 5,000 on Immune Response Biomarker Profiling ProtoArray) as compared to internal array controls. Unshaded cells present the number of shared autoantibodies with positivity between the two groups. The number of autoantibodies shared exclusively or between the two groups (for example, those autoantibodies with positivity in groups A and B but not C or D) is presented in brackets.

Table 4

Relative serum anti-glutathione S-transferase alpha titers in mining populations.

Type of mining	N	anti-GSTA1 (median, IQR)	Sex	Prevalent malaria
emerald	91	933 (621-1561)	male=63%	yes=2%
diamond	57	558 (332-776)	male=89%	yes=37%
gold	223	5090 (3162-8752)	male=67%	yes=54%

IQR: inter-quartile range

Table 5

Odds ratios of high anti-glutathione S-transferase alpha titer in mining populations. Emerald miners were used as the referent group.

Population	OR	95% CI
Model 1 ^a		
Diamond miners	1.1	0.2, 6.6
Gold miners	89.6	27.2, 294.6
Model 2 ^b		
Diamond miners	1.0	0.2, 6.4
Gold miners	150.5	38.6, 586.0

^a simple logistic regression.

^b simple logistic regression adjusted for sex and malarial status.