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Interaction between a Mixture of Heavy Metals (Lead, Mercury, Arsenic, Cadmium, Manganese, Aluminum) and *GSTP1, GSTT1,* and *GSTM1* in Relation to Autism Spectrum Disorder

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Abstract

Background: Exposure to many environmental chemicals, including metals, often does not occur in isolation, hence requires assessment of the associations between exposure to mixtures of chemicals and human health.

Objectives: To investigate associations of a metal mixture of lead (Pb), mercury (Hg), arsenic (As), cadmium (Cd), manganese (Mn), and aluminum (Al) in children with autism spectrum disorder (ASD), additively or interactively with each of three glutathione S-transferase (GST) genes (*GSTP1*, *GSTT1*, and *GSTM1*).

Method: Using data from 266 case-control pairs of Jamaican children (2–8 years old), we fitted negative and positive generalized weighted quantile sum (gWQS) regression models to assess the aforementioned associations.

Results: Based on additive and interactive negative gWQS models adjusted for maternal age, parental education, child's parish, and seafood consumption, we found inverse associations of the overall mixture score with ASD [MOR (95% CI): 0.70 (0.49, 0.99); $P < 0.05$) and [MOR (95%CI): 0.46 (0.25, 0.84); $P = 0.01$], respectively. In an unadjusted negative gWQS model, we found a marginally significant interaction between *GSTP1* and a mixture of three metals (Pb, Hg, and Mn) ($P = 0.07$) while the association was no longer significant after adjustment for the same covariates ($P = 0.24$).

Conclusions: Differences in diet between ASD and control groups may play a role in the inverse associations we found. The possible interactive association between Mn and *GSTP1* in ASD based on gWQS is consistent with our previous reports. However, possible interaction of *GSTP1* with Pb and Hg in ASD requires further investigation and replication.

Keywords

Autism Spectrum Disorder; glutathione S-transferase (GST) genes *(GSTP1, GSTT1,* and *GSTM1)*; Mixture analysis; Generalized weighted quantile sum regression (gWQS); Heavy metals; Jamaica

1. Introduction

Exposure to heavy metals in early childhood has adverse influences on neurodevelopment (Rossignol, Genuis, & Frye, 2014; Chen, Miah, & Aschner, 2016; Claus et al., 2012; Ha et al., 2009; Claus, Coull, & Wright, 2014). For example, early childhood exposure to lead (Pb), mercury (Hg), arsenic (As), cadmium (Cd), and aluminum (Al) are associated with Autism Spectrum Disorder (ASD) (Mohamed et al., 2015; Yasuda & Tsutsui, 2013; Fido & Al-Saad, 2005; Kern, Grannemann, Trivedi, & Adams, 2007; Windham, Zhang, Gunier, Croen, & Grether, 2006; Yorbik, Kurt, Hasimi, & Ozturk, 2010; Blaurock-Busch, Amin, Dessoki, & Rabah, 2012; Soden, Lowry, Garrison, & Wasserman, 2007; Blaurock-Busch, Amin, & Rabah, 2011; Clark, Vandermeer, Simonetti, & Buka, 2010; Obrenovich, Shamberger, & Lonsdale, 2011; Adams, Romdalvik, Ramanujam, & Legator, 2007; DeSoto & Hitlan, 2007; Lakshmi Priya & Geetha, 2010). However, most of these findings are related to assessing the role of a single environmental exposure on ASD based on additive models considering a single exposure at a time. Several studies have linked oxidative stress with ASD (Chauhan & Chauhan, 2006; Coles & Kadlubar, 2003; James et al., 2006; James et al., 2004; James, 2008). Glutathione-S-transferase (GST) enzymes catalyze the conjugation of glutathione (GSH) to xenobiotic compounds, including heavy metals and metalloids such as Pb, Cd, Hg, Al, Mn, and As (Shahid et al., 2014; Patra, Rautray, & Swarup, 2011a; Patra, Rautray, & Swarup, 2011b; Grotto et al., 2010), and thus play an important role in protecting against oxidative stress (Sharma, Yang, Sharma, Awasthi, & Awasthi, 2004; Jan et al., 2015).

In addition, a limited number of these studies were able to adjust their findings by potential confounders such as diet. It is well known that children with ASD have a higher incidence of gastrointestinal (GI) problems (70% vs. 28%) (Valicenti-McDermott et al., 2006), such as, constipation (33.9% vs. 17.6%) (Horvath & Perman, 2002), which may result in food selectivity for children with ASD compared to typically developing (TD) children (Ibrahim, Voigt, Katusic, Weaver, & Barbaresi, 2009). Schreck *et al.* (2006) and Cermak *et al.* (2010) reported lower levels of consumption of fruits and vegetables by children with ASD compared to TD children (Schreck & Williams, 2006; Cermak, Curtin, & Bandini, 2010). Rahbar et al. also reported that the consumption of some vegetables and fruits is associated with higher levels of blood arsenic concentrations, which suggests that the above factors could play a major role in confounding of the association between blood arsenic concentrations and ASD (Rahbar et al., 2012). In order to obtain unbiased results when assessing associations between blood metal concentrations and ASD, it is important to identify and adjust for potential confounding variables in the multivariable analysis.

Since individuals are exposed to multiple chemicals in the environment simultaneously and these exposures are potentially correlated, mixture analysis methods such as Weighted Quantile Sum (WQS) (Czarnota, Gennings, & Wheeler, 2015a; Carrico, Gennings, Wheeler, & Factor-Litvak, 2015) have recently been proposed that account for potential correlations among the exposures that occur together and perhaps simultaneously. Analysis of environmental exposure data without consideration of influences from a mixture of chemicals could result in erroneous findings.

Limited information is available regarding the comparison of levels of environmental exposure in children with and without ASD from Low-Middle Income Countries (LMIC). Exposure to environmental metals and metalloids such as Pb, Hg, As, Cd, Al, and manganese (Mn) may be associated with ASD because the levels of these metals and metalloids could be higher in some developing countries.

Jamaica provides a unique opportunity to study the health associations between exposure to the aforementioned six metals and ASD, as it has unusually high levels of these six metals. For example, mean Pb levels in Jamaican soil (44mg/kg) may be four times that in some other parts of the world (average Pb level = 10mg/kg) (Knight, Kaiser, Lalor, Robotham, & Witter, 1997). Wright et al. investigated concentrations of nine residual metals in some Jamaican foods and reported that sweet potatoes had the highest concentrations of Pb (0.31mg/kg) (Wright, Jones, & Omoruyi, 2012). Lalor et al. reported a mean blood Pb concentration of 7.3μg/dL in several groups of Jamaican school children in corporate areas of Kingston and St. Andrew (Lalor, Vutchkov, & Bryan, 2007). In addition, Jamaica has very specific sources of exposure to Hg, including fish and seafood consumption (Knight et al., 1997; Lalor, 1996; Howe, Fung, Lalor, Rattray, & Vutchkov, 2005), and there are published reports indicating that high levels of As were found in the soil of central Jamaica (Howe et al., 2005; Lalor, 1996) as well as in the drinking water (Lalor, Rattray, Simpson, & Vutchkov, 1999). Levels of As in agricultural soil and water could affect fruits, vegetables, and other foods (Lalor et al., 1999).

Moreover, Jamaica provides a unique opportunity to study the health associations of Cd exposure, as it has an unusually high level of this naturally occurring metal (Lalor, 2008). Concentrations in soil are ubiquitous and exceptionally high in certain parishes, with some greater than 900mg/kg (Lalor, 2008; Wright, Rattray, Lalor, & Hanson, 2010). Concentrations of Cd in various Jamaican crops including fruit, legumes, leafy vegetables, root vegetables, and especially yams are significantly higher than those seen in other countries (Howe et al., 2005; Lalor, 2008). A worldwide comparison by the International Atomic Energy Agency also reported that levels of Mn found in Jamaican soil are approximately twice the average reported for soils in other countries (Lalor, 1996). Jamaica's soil has higher levels of Al than most countries around the world, with the highest Al concentrations near the main bauxite-mining belt and in small areas on the east and the west coasts (Lalor, 1996). Findings from our previous studies also indicate that Jamaican children aged 2–8 years have mean blood Pb concentrations 2–3 times higher, concentrations of Hg 3 times higher, and As concentrations 4.5 times higher than children in North America (Rahbar et al., 2012; Rahbar et al., 2013; Rahbar et al., 2015a).

Since 2009, our research team at the University of Texas Health Science Center at Houston (UTHealth) has collaborated with the team at the University of the West Indies (UWI) in Jamaica to investigate the role of the six aforementioned heavy metals, and their potential interaction with the GST genes (*GSTP1*, *GSTT1*, and *GSTM1*) in relation to ASD in Jamaica. So far, our collaboration has led to reporting the absence of associations between ASD status and each of the six metals in additive models (Rahbar et al., 2012; Rahbar et al., 2013; Rahbar et al., 2014c; Rahbar et al., 2014a; Rahbar et al., 2016). We have also reported a significant synergistic association of blood manganese concentrations (BMC) and *GSTP1*

in relation to ASD, indicating that among children who had the Ile/Ile genotype for *GSTP1*, those with BMC ≥ 12μg/L had about 4 times higher odds of ASD than those with BMC< 12μg/L, ($P = 0.03$) (Rahbar et al., 2015b). In addition, we also reported a significant interaction between *GSTP1* and ASD status in relation to concentrations of Al and As in blood of Jamaican children (Rahbar et al., 2016; Rahbar et al., 2014d).

In a more recent paper, we have demonstrated that the interaction between Mn and *GSTP1* in relation to ASD remained significant with similar magnitude of associations after adjusting for the mixture of four other metals (Pb, Hg, As, and Cd) based on an estimated mixture score using weighted quantile sum (WQS) regression models (Rahbar et al., 2018). However, since the current version of WQS does not allow assessment of interactions between/among exposures (e.g., interaction between a mixture of metals and GST genes), our team developed a generalized WQS (gWQS) method that handles such interactions (Lee et al., 2019). The advantages of Lee *et al.*'s gWQS method also include accounting for possible correlation due to matching of ASD cases and TD controls and for having left censored data due to a portion of metal concentrations being below the limit of detection (LoD) (Lee et al., 2019). The main objective of this paper is to apply the gWQS algorithm developed by Lee *et al.* (Lee et al., 2019) to assess the role of environmental exposure to a mixture of the six metals (Pb, As, Mn, Hg, Cd, and Al) and each of three GST genes (*GSTP1*, *GSTT1*, and *GSTM1*) and their possible interactions in relation to ASD and to compare our findings with those obtained from comparable Conditional Regression Models (CLRs).

2. Methods

2.1 General description of study design and populations

The Epidemiological Research on Autism in Jamaica (ERAJ) is an age- and sex-matched case-control study (children age 2–8 years) that began enrollment in December 2009, investigating whether environmental exposures to six metals are associated with ASD. The recruitment and assessment of ASD cases and controls has been described previously (Rahbar et al., 2012; Rahbar et al., 2013; Rahbar et al., 2014a; Rahbar et al., 2014d; Rahbar et al., 2014b; Rahbar et al., 2014c; Rahbar et al., 2016; Rahbar et al., 2018). In brief, children included in the UWI Jamaican Autism Database, who were previously identified as being at risk for ASD based on Diagnostic Statistical Manual of Mental Disorders (DSM-IV-TR) criteria (APA, 2000) and the Childhood Autism Rating Scale (CARS) (Schopler, Reichler, DeVellis, & Daly, 1980), were invited to participate for reassessment of their ASD status for this study. We administered the Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2000), Autism Diagnostic Observation Schedule™, Second Edition (ADOS-2) (Lord et al., 2012) and the Autism Diagnostic Interview-Revised (ADI-R) (Rutter, Le, & Lord, 2003) to confirm the diagnosis of ASD. Established cutoff points were utilized for the ADOS, ADOS-2 and ADI-R (Rutter et al., 2003; Lord et al., 2000; Lord et al., 2012). Each ASD case was confirmed based on both ADI-R and all three domains in ADOS or ADOS-2. For each confirmed ASD case, an age- and sex-matched TD control (within six months of the matched ASD case) was identified from schools and well-child clinics. For ascertainment of TD (based on age), we administered the Lifetime or Current Forms of the

Social Communication Questionnaire (SCQ) (Rutter, Bailey, & Lord, 2003) to the parents/guardians of potential TD control children to rule out symptoms of ASD. We set the criteria for including children in the TD control group as having a SCQ score of 0–6. (Mulligan, Richardson, Anney, & Gill, 2009).

We administered a socioeconomic status (SES) questionnaire to assess demographic characteristics, education level of the parents, and potential exposure to metals through the parents' occupations or other sources. We also administered a food frequency questionnaire that captures possible dietary exposure to metals. Specifically, we collected information about the type and frequency of seafood consumed weekly by the child. We considered salt water fish, fresh water fish (pond fish, tilapia), sardine or mackerel (canned fish), tuna (canned fish), salt fish (pickled mackerel), shellfish (lobsters, crabs), and shrimp. Similarly, we collected information about the type and the frequency of consumption of fruits and vegetables that were classified into the following categories: (1) two classes of root vegetables [(yam, sweet potato, or dasheen) and (carrot, or pumpkin)]; (2) three classes of leafy vegetables [(lettuce), (callaloo, broccoli, or pak choi), and (cabbage)]; (3) legumes (string beans); and (4) three different fruits (tomatoes, ackee, or avocado). We also collected information regarding the frequency and consumption of meat/organ meat, dairy products, eggs, grain/starches, peas, beans, nuts, juices/soft drinks, and sources of drinking and cooking water. For analysis, we categorized the frequency of food consumption into two levels (never consumed or consumed). At the end of the interview, 2–3 mL of whole blood was collected from each child for assessment of exposure to the metals. In addition, 2–3 mL of blood was collected for genotyping. For this study, we used data from 532 children 2–8 years old (266 case-control pairs) from our autism project in Jamaica.

2.2 Methods for assessment of the metals exposure

For this study, the blood samples were shipped to the Michigan Department of Health and Human Services (MDHHS) in Lansing, Michigan, USA, for assays of trace metals (Pb, Hg, As, Cd, Mn, and Al). The Trace Metals Lab at MDHHS is certified by the Centers for Disease Control and Prevention (CDC). Venous whole blood samples were diluted and analyzed using a PerkinElmer Elan DRCII inductively-coupled plasma mass spectrometer (PerkinElmer, Waltham, MA) for trace metal analyses. The methods for analysis of the six metal concentrations in blood by MDHHS have been described previously (Rahbar et al., 2012; Rahbar et al., 2013; Rahbar et al., 2014a; Rahbar et al., 2014d; Rahbar et al., 2014b; Rahbar et al., 2014c; Rahbar et al., 2016; Rahbar et al., 2018).

Blood metal concentrations were reported by MDHHS as undetectable if they were below the LoD. The LoD of the six heavy metals analyzed for our study were reported in our previous paper (Rahbar et al., 2018). In this study, the percentage of each blood metal concentrations below the LoD was as follows: Hg (16.7%), As (38.9%), Cd (55.5%), Al (29.8%), Pb (0.0%), and Mn (0.0%).

2.3 Genetic assessment and analysis

Methods for genetic analysis of the *GSTP1* Ile105Val polymorphism (rs1695; C_3217198_20) have been described in detail previously (Rahbar et al., 2014d; Rahbar et

al., 2016). Whole blood was processed by the CARIGEN lab at UWI and then shipped to the UTHealth School of Public Health (UTSPH) Human Genetics Center (HGC) Laboratory in Houston, Texas. Genomic DNA was isolated from buffy coat using the Gentra PUREGENE Blood Kit (Qiagen, N.V., Venlo, The Netherlands). Two regions of the *GST* gene were amplified in independent TaqMan Copy Number Assay reactions; *GSTM1* Assay ID: Hs02575461_cn and *GSTT1* Assay ID: Hs00010004_cn (www.thermofisher.com). Each quantitative PCR reaction contained 10 ng of gDNA, TaqMan Genotyping Master Mix, TaqMan Copy Number Assay, and TaqMan Copy Number Reference Assay (RNaseP) in a 5μL reaction in accordance with instructions provided by the manufacturer. Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The real-time QuantStudio and 12K Flex Software v1.2.2 was used to assay each sample in quadruplicate. Samples were normalized to RNaseP and averaged to obtain a single ΔCt value (FAM dye Ct – VIC dye Ct) which was imported into CopyCaller Software v2.1 (www.thermofisher.com) to determine the copy number of each genomic DNA target.

The choice of assays used for the three genes (*GSTM1*, *GSTT1*, and *GSTP1)* was related to the type of polymorphism that was investigated. The genetic variant in *GSTP1* is a single nucleotide polymorphism (SNP) that results in an amino acid change at position 105 of the encoded protein (Ile105Val). There are three different possible genotypes and all of them can be distinguished from each other: Ile/Ile, Ile/Val, and Val/Val. We used the frequencies of all 3 genotypes in the TD control group from Jamaica to calculate genotype frequencies and to determine whether Hardy-Weinberg equilibrium expectations were met using the chi-square test in the TD control group. We analyzed the *GSTP1* gene using different genetic models, including the dominant (Val/* vs Ile/Ile) and the co-dominant model (Ile/Ile, Ile/Val, and Val/Val).

For the *GSTM1* and *GSTT1* genes, a deletion/insertion polymorphism was evaluated. Since the assay does not distinguish between a normal homozygote (I/I) and a heterozygote (I/D), we considered only a recessive model using a binary variable to represent the genotype: I* and DD. However, the inability to calculate accordance with Hardy-Weinberg equilibrium expectations for *GSTM1* and *GSTT1* is a limitation of the methods used.

2.4 Statistical analysis

2.4.1 Comparison of characteristics between ASD cases and typically developing (TD) control groups—

We compared the distributions of demographic characteristics, socioeconomic characteristics, frequencies of the *GSTP1*, *GSTT1*, and *GSTM1* genotypes, and the six blood metal concentrations between ASD cases and TD controls using CLR. models. Details regarding the description of the exposure variables have been reported previously (Rahbar et al., 2012; Rahbar et al., 2014d).

To minimize the influence of measurements below LoD and/or skewed distributions of metals in our study, concentrations of each of the six metals that were below the LoD were replaced by the LoD for that metal divided by the square root of two (LoD/√2) (Hornung & Reed, 1990; Meeker, Sathyanarayana, & Swan, 2009). Furthermore, because a sizeable portion of concentrations were below LoD for Hg, Al, As, and Cd, we used appropriate random effect quantile regression models (for Cd, at 75th percentile and for Hg, Al, and As

at the median concentration) to compare ASD cases and TD control groups while controlling for the potential clustering effect of the matched pairs. Since the distributions of blood Pb concentrations were skewed, we transformed these data using the natural logarithm (ln) to produce approximately normal distributions. We used univariable General Linear Models (GLM) that controlled for the matched pairs to calculate geometric means for Pb but arithmetic means for Mn for both ASD cases and TD controls groups, because blood Mn concentrations had an approximately normal distribution for both ASD cases and TD controls.

2.4.2 Assessment of correlation between all pairs of blood metal concentrations (overall and by genotypes)—We initially assessed pairwise correlations between all pairs of the log transformed metal concentrations. For Mn, pairwise correlations with the other five log-transformed concentrations were assessed. We also assessed the same pairwise correlations by *GSTP1*, *GSTT1*, and *GSTM1* genotypes.

2.4.3 Reassessment of associations of individual metal concentrations with ASD status—Previously, we have used GLMs without (for Mn) or with the log-transformed metal concentrations of each metal (Pb, Hg, Al, and As) as the dependent variable to investigate the possible additive association of individual metals with ASD status. In all GLMs, we also controlled for the clustering effect of matching by including an appropriate number of dummy variables that represented the matched pairs (e.g., 265 dummy variables for 266 matched pairs). Considering the availability of a much larger data set for each metal (2–3 times larger), we re-evaluated the GLM models for Pb and Mn adjusted for potential confounders (based on our previous publications for each of these two metals with smaller sample sizes) (Rahbar et al., 2014b; Rahbar et al., 2015b), and calculated mean concentrations by ASD status along with *P*-values for these two metals. However, since a sizeable portion of blood concentrations for Hg, Al, As, and Cd were below the LoD (i.e., ranged from 16.7% for Hg to nearly 55% for Cd), we compared the 75th percentile of blood Cd concentrations (Rahbar et al., 2014c) and median concentrations for Hg, Al, and As of ASD cases and TD controls using quantile regression models with random effects. The random effects were chosen to account for possible correlation between matched case-control pairs with respect to blood As, Al, Hg, and Cd concentrations and these models were adjusted for appropriate potential confounders that were reported previously (Rahbar et al., 2016; Rahbar et al., 2012; Rahbar et al., 2013; Rahbar et al., 2014a).

2.4.4 Potential confounders and covariates—As mentioned earlier, for assessing associations between metals and ASD, various factors such as diet could serve as potential confounders. Because of their importance, in the following we provide a list of variables that were identified as potential confounding variables or were used as covariates if they changed the regression coefficient by >10% for the binary metal variable or from literature including our previous publications that assessed the association between the individual metals and ASD using data from our ERAJ study (Rahbar et al., 2016; Rahbar et al., 2012; Rahbar et al., 2013; Rahbar et al., 2014a; Rahbar et al., 2014b; Rahbar et al., 2014c), For ease of access, we also reported this list in the footnote of Table 2. The covariates that were

considered for inclusion in models with interaction between an individual GST gene and the metals were based on the literature including our previous papers related to the synergistic association of Mn and *GSTP1* (Rahbar et al., 2015b). Specifically, for all the adjusted CLRs and all adjusted gWQS models, we used four binary covariates, maternal age (<35 vs. ≥35 years), parental education levels (both parents had education up to high school vs. at least one of the parents had education beyond high school), parish at child's birth (Kingston parish vs. other parishes), and consumption of seafood (≤ 6 meals per week vs. > 6 meals per week).

At all stages of model building we were mindful of potential correlations between any pairs of independent variables or covariates that had the potential for multicollinearity and these were excluded from analysis.

2.4.5 Interactive associations of the metal concentrations and each of the GST genes in relation to ASD using CLR models—Since a sizeable portion of concentrations were below LoD for Hg, Al, As, and Cd, an *a priori* a decision was made to minimize the impact of replacement of concentrations below the LoD with LoD/√2. Specifically, for the analyses in this section, we considered two thresholds, 10–35% below LoD (medium) and 35–65% below LoD (high) (Canales, Wilson, Pearce-Walker, Verhougstraete, & Reynolds, 2018), for dichotomizing the metal concentrations as below or above their respective LoDs (≥LoD vs. <LoD). The reason for choosing these thresholds was, on one hand, to ensure at least 10% of the overall concentrations for at least 53 (10%*532 ≈ 53 children were in each of the two categories for analysis, while, on the other hand, the upper threshold of 65% below the LoD was selected to ensure at least 35% (i.e., 100% – 65% = 35%) of concentrations fell in this category that indicate a measurable level of exposure in children who participated in this study. Based on these criteria Hg and Al had a medium percentage, and As, and Cd has a high percentage below their respective LoDs. Therefore, Hg and Al were dichotomized as binary variables (≥LoD vs. <LoD).

Although As and Cd had 38.9% 55.5% of their concentrations below their LoDs, we also decided to investigate the potential interaction of each of these two with each of the GST genes in CLR models, with an understanding that their inclusion in the multivariable CLR would be dependent on our finding from the sensitivity analysis that compared the final multivariable CLR models with and without inclusion of either As or Cd or both AS and Cd in the CLR models. For this reason, As and Cd were also dichotomized into binary variables (≥LoD vs. <LoD).

Mn and Pb did not have any concentrations below LoD, but since the assumption of linearity on the logit scale (ln of odds of ASD given concentrations of each metal) was violated for blood metal concentrations of Pb and Mn, we did not analyze exposure to these metals as continuous variables in CLR models. Instead, we categorized Pb into 4 quartiles to better align this variable with the way it was analyzed as a mixture using gWQS regression. However, blood Mn concentrations was dichotomized as ≥75th percentile and <75th percentile in order to be able to compare with our previous findings of a significant interaction between *GSTP1* and Mn (Rahbar et al., 2015b).

2.4.6 Mixture analysis of exposure to six metals using generalized Weighted Quantile Sum (gWQS) regression models—

Since the correlation coefficients between each pair of metal concentrations ranged between 0.05–0.53, simultaneous inclusion of the six metal concentrations as independent variables in a regression model may cause some level of multicollinearity, which could result in variance inflation and unstable *P*-values. WQS regression, (Carrico et al., 2015) which is shown to perform well under such situations (Czarnota et al., 2015; Gennings et al., 2013; Czarnota, Gennings, & Wheeler, 2015b), can estimate a body burden index that identifies important contributors to the associations between a mixture of correlated concentrations of the six metals and ASD (Czarnota et al., 2015b). We used generalized WQS regression (Carrico et al., 2015; Czarnota et al., 2015; Gennings et al., 2013; Czarnota et al., 2015b) models, which construct a weighted index representing the mixture association of all six metal concentrations. The relative contribution of each metal to the overall mixture in association with ASD was tested, and identified by the weights that are empirically determined through bootstrap samples such that we can better understand the association of individual exposures to each metal as well as their overall mixture association with ASD status. As WQS regression provides a unidirectional evaluation of a mixture association, positive (non-negative association) or negative (non-positive association) (Carrico et al., 2015), which is separately evaluated with respect to an outcome variable (i.e., ASD status), we constructed models with positive and negative associations separately on the weighted index. This enabled us to make analysis appropriate in a setting where the goal is to properly identify both magnitude and direction of the individual exposure associations on a health outcome. Considering that our goal is also to test for possible interactive associations between blood concentrations of each metal and GST genes *(GSTP1, GSTT1,* and *GSTM1)* in relation to ASD status, we utilized the interactive gWQS estimation algorithms developed by Lee, *et al.* (Lee et al., 2019) that can explore interactive associations by estimating genotype-specific weights of individual metals while accounting for the potential clustering effect of matched pairs of ASD cases and TD controls, which cannot be done by the original WQS method (Carrico et al., 2015; Czarnota et al., 2015; Gennings et al., 2013; Czarnota et al., 2015b). Specifically, the metal concentrations are scored into quartiles (0, 1, 2, and 3) and used in an interactive gWQS algorithm (Lee et al., 2019). The algorithm determines the relevant contribution of each of the six metal concentrations to the association between the gWQS index and ASD status and their potential interactions with GST genotypes by estimating genotype-specific weights that are empirically determined through bootstrap sampling. This interactive gWQS method also enabled us to conduct comparisons between models with and without interactions, to assess the statistical significance of interactive associations. Further details regarding the interactive gWQS method are available elsewhere (Lee et al., 2019). We also adjusted the results from the multivariable gWQS models by maternal age, parental education levels, parish at child's birth, and consumption of seafood as covariates.

2.4.7 Comparison of the final interactive gWQS model with multivariable CLR

—Since the overall (as well as stratified by genotypes) correlations between pairs of metal concentrations were low to moderate, we also fitted a multivariable CLR with the same independent variables that were included in the final interactive gWQS model. Statistical significance of all the interactions in the interactive CLR model were assessed based on the

difference between −2 log likelihood of the additive CLR and the interactive CLR. Acknowledging that some lower level of multicollinearity may be caused by the correlated metal concentrations, this allows comparison of our findings from the gWQS method with those obtained from the CLR. As mentioned earlier in section 2.4.5, we also conducted sensitivity analysis in the final multivariable CLR models with and without inclusion of one or both binary Cd and As variables.

All analyses that involved GLMs or CLRs were performed using SAS version 9.4 (SAS Institute Inc., 2013). For gWQS mixture analyses, we used the estimation algorithms in R (R Core Team, 2015) developed by Lee *et al.* that are provided in the Appendix of their paper (Lee et al., 2019). All statistical hypotheses were tested at 0.05 level of significance.

3. Results

The mean age of ASD cases and TD controls was 5.3 years. About 81% of the ASD cases and TD controls were male. Nearly all of the ASD cases (95.5%) and TD controls (97%) were Afro-Caribbean. Similarly, approximately 96.5% of both parents in both ASD cases and TD control groups were Afro-Caribbean. A higher proportion of both the mothers (18.9%) and fathers (46%) of ASD cases were age 35 or greater at the time of the child's birth compared to the mothers (12.3%) and fathers (31.0%) of TD controls. Similarly, the mothers (48.5%) and fathers (41.4%) of ASD cases had higher levels of education than the mothers (37.6%) and fathers (22.3%) of TD controls. ASD cases also had a higher SES than TD controls, with 56.4% of case families owning a car versus 39.9% of car ownership by control families.

Compared to TD controls, ASD cases had lower geometric mean blood concentrations of Pb (1.92μg/dL vs. 2.34μg/dL; $P < 0.01$) and median concentrations for Hg (0.64μg/L vs. 0.81μg/L; $P = 0.01$). In contrast, no significant differences were detected between ASD cases and TD controls with respect to median blood concentrations of As and Al, or the arithmetic mean blood concentration of Mn (all $P > 0.55$). Based on the quantile regression analysis, there was no significant difference between the ASD case and TD control groups with respect to the 75th percentile of blood Cd concentrations (0.2μg/L for ASD cases vs. 0.2μg/L for TD controls, $P = 1.00$).

There were no significant differences in genotype frequencies for *GSTP1*, *GSTT1*, or *GSTM1* between ASD cases and TD controls (all three $P > 0.11$). Furthermore, the *GSTP1* genotype frequency in the TD controls was in agreement with Hardy-Weinberg equilibrium expectations ($P = 0.28$).

The percentage of blood metal concentrations below the LoD for each metal is 0%, 16.7%, 38.9%, 55.5%, 29.8% and 0%, for Pb, Hg, As, Cd, Al, and Mn, respectively. The overall and genotype stratified pairwise correlation coefficients between each pair of the six metals were calculated. The overall correlation coefficients between all pairs of blood metal concentrations ranged from 0.05 to 0.53. Arsenic was the most consistently correlated with the other metals across all possible *GSTP1* genotypes, which ranged from r = 0.50 to 0.55 for Al, r = 0.11 to 0.32 for Pb, r = 0.34 to 0.48 for Hg, r = 0.33 to 0.39 for Cd, and r = 0.10

to 0.19 for Mn. Aluminum was the most consistently correlated with the other metals across all possible *GSTM1* genotypes, which ranged from r = 0.53 to 0.54 for As, r = 0.22 to 0.23 for Cd, r = 0.10 to 0.20 for Pb, r =0.12 to 0.15 for Hg, r = 0.001 to 0.11 for Mn, and r = 0.34 to 0.47 for Cd. Arsenic was the most consistently correlated with the other metals across all possible *GSTT1* genotypes, which ranged from r = 0.49 to 0.54 for Al, r = 0.36 to 0.40 for Hg, r = 0.36 to 0.42 for Cd, r = 0.14 to 0.29 for Pb, and r = 0.11 to 0.17 for Mn. Additional details regarding these correlations are provided in Table S.1 as part of the supplementary material.

After adjusting for potential confounders that we selected from our previous publications for each of these metals (Rahbar et al., 2016; Rahbar et al., 2012; Rahbar et al., 2013; Rahbar et al., 2014a; Rahbar et al., 2014b; Rahbar et al., 2014c) and are reported in the footnote of Table 2, there were no significant differences between ASD cases and TD controls with respect to geometric mean blood concentrations of Pb, and median concentrations for Hg, As, and Al and the arithmetic mean blood concentration of Mn (all $P > 0.30$). Similarly, after adjusting for potential confounders in the quantile regression analysis, there was no significant difference between the ASD case and TD control groups with respect to the 75th percentile of blood Cd concentrations (0.2μg/L for ASD cases and 0.2μg/L for TD controls, $P = 0.67$). These findings indicate lack of additive association between each of the six metals and ASD (all $P > 0.30$).

In multivariable CLR, we also assessed potential interactive associations between each of the blood metal concentrations and each of the *GST* genes in relation to ASD status. Those findings that were related to Mn, Pb, and Hg (metals with % below LoD <20%) or resulted in *P*-values less than 0.25for the overall interaction term are shown in Table 3. Other results from these analyses are provided in Tables S.2, S.3, and S.4, as part of the supplementary material.

Specifically, in co-dominant models for *GSTP1*, the interactions between *GSTP1* and blood concentrations of Pb, Hg, As, Al, or Cd were not statistically significant (all $P > 0.12$). However, the overall interaction between *GSTP1* and blood concentrations of Mn was marginally significant ($P = 0.09$). In addition, considering blood concentrations of Mn <75th percentile as the referent category, we found that among children with the Ile/Ile genotype, the odds of having blood concentrations of Mn ≥75th percentile (12μg/L) in ASD cases was 1.81 times that of the TD control group, [matched odds ratio (MOR) (95% CI) = 1.81 (0.88, 3.70)], while among children with the Ile/Val or Val/Val genotypes, the odds of having blood concentrations of Mn ≥75th percentile in ASD cases were lower than in the TD control group, [MOR (95% CI) = 0.84 (0.48, 1.48), and MOR (95% CI) = 0.57 (0.22, 1.44)], respectively. Similarly, in the dominant models for *GSTP1*, the interactions between *GSTP1* and blood concentrations of Pb, Hg, As, Al, or Cd were not statistically significant (all $P > 0.08$). In contrast, we found a significant ($P = 0.03$) interaction between binary blood Mn concentrations and *GSTP1.* Specifically, considering blood concentrations of Mn <75th percentile as the referent category, we found that among children with the Ile/Ile genotype, the odds of having blood concentrations of Mn ≥75th percentile (12μg/L) in ASD cases was 1.87 times that in the TD control group, [MOR (95% CI) = 1.87 (0.92, 3.38)], while among children with the Ile/Val or Val/Val genotype, the odds of having blood concentrations of Mn

≥75th percentile in ASD cases was 0.77 times that in the TD controls, [MOR (95% CI) = 0.77 (0.47, 1.25)]. Additional details regarding these findings are provided in Table S.2, as part of the supplementary material.

In CLR models with ASD status as a dependent variable and the categorized blood metal concentration and *GSTM1* as independent variables, the interactions between *GSTM1* and blood concentrations of Pb, Hg, Cd, Al, As or Mn were not statistically significant (all $P > 0.40$). Additional details regarding these findings are provided in Table S.3, as part of the supplementary material. Similarly, in CLR models with ASD status as a dependent variable and the binary blood metal concentration and *GSTT1* as independent variables, the interactions between *GSTT1* and blood concentrations of Pb, Hg, As, Mn and Cd were not statistically significant (all $P > 0.07$). Although the interactions between *GSTT1* and blood concentrations of Al was significant ($P = 0.04$), none of the two genotype-specific *P*-values were statistically significant at the 0.05 level (both $P > 0.09$). Additional details regarding these findings are provided in Table S.4, as part of the supplementary material.

Subsequently, using both univariable and multivariable mixture analysis, we evaluated the association of each metal with ASD status when the overall association of the six metals was modeled using the gWQS algorithm (Lee et al., 2019) that takes into account the possible interactions between each of the three GST genes and a mixture of metals while accounting for correlations among the metals as well as censoring due to a portion of blood metal concentrations being below their respective LoDs.

Table 4 displays the unadjusted and adjusted associations between a mixture of metals and ASD status based on additive and interactive gWQS models under the framework of CLR. Negative and positive associations of the mixture were assessed, reporting both the overall associations (MOR and the 95% CI) and the individual contribution of each metal to the overall mixture association (weights assigned to each metal in the mixture). An *a priori* rule was used to identify a metal that made a strong contribution to the mixture index through its association with ASD based on a weight in the additive model with all six variables that was ≥ 0.166 (1/6). This is because if the 6 metals were equal contributors to the mixture then the weight of each of the six metals is expected to be 1/6 = 0.166. Similarly, in an interactive mixture model with 12 variables with their corresponding weights (a total of 12 additive and interactive terms), a metal with a weight of ≥ 0.083 (1/12) was considered to be a strong contributor. The choice of cut point may also be affected by the number of variables, correlation structure, signal strength, and other factors; further discussion regarding a suitable cutoff value is available elsewhere (Lee et al., 2019).

In the *positive* additive WQS models after controlling for maternal age, parental education levels, parish of child's birth and consumption of seafood as potential confounders, the contributions of As (weight of 0.46), Mn (weight of 0.26) and Al (weight of 0.22), to a positive association between the mixture of metals and ASD status, were considered stronger than the contributions of Pb, Hg, and Cd which had estimated weights of ≤ 0.03). While the additive WQS model provides one weight per metal, an interactive gWQS model (Lee et al., 2019) provides estimated weights of metals for each of the three *GSTP1* genotypes separately. Based on the positive interactive gWQS model, the estimated weights

of Mn for Val/Val, Ile/Val, and Ile/Ile were 0.04, 0.02, and 0.17, respectively, indicating that the contribution from Mn to the positive mixture association with ASD noticeably differed by *GSTP1* genotype, which could have been missed in additive WQS models. We also found that the estimated weights of Hg and Pb varied by *GSTP1* genotype. Specifically, while there was almost no contribution (0.001) from Hg to the positive mixture association on ASD in the additive WQS model, the interactive gWQS model revealed that the estimated weights of Hg for Val/Val, Ile/Val, and Ile/Ile were 0.001, 0.05, and 0.15, respectively. Similarly, in the interactive gWQS model the estimated weight of Pb for children with the Val/Val genotype was 0.13, but minimal contributions of Pb were found in children with the Ile/Val (0.0004) and Ile/Ile genotypes (0.001), which could not be captured in the additive WQS model in which the estimated weight of Pb was 0.03. A strong contribution found from As (0.46) and Al (0.22) in the additive WQS model remained strong in the interactive gWQS model (i.e., 0.27 and 0.14 for As and Al, respectively).

We explored *negative* mixture associations of metals with ASD status as well in order to complete the assessment of the association of the heavy metals in individuals with different *GSTP1* genotypes in relation to ASD. In the negative gWQS model, we also found that the association of the three metals (Pb, Hg, and Mn) with ASD appeared to differ by *GSTP1* genotype. Specifically, while we found a relatively small weight for Mn (0.02) in the negative additive unadjusted WQS model, the weight of Mn for children with the *GSTP1* Val/Val genotype estimated from the interactive gWQS model was 0.19, and the smallest contribution from Mn to the negative mixture association with ASD was identified for the *GSTP1* Ile/Ile genotype (0.02), which aligns with the findings from the positive gWQS model that the *GSTP1* Ile/Ile genotype group had the highest weight (0.15) for Mn. The findings from both positive and negative gWQS models suggested that only children with the *GSTP1* Ile/Ile genotype had a strong association between higher Mn concentration and ASD status. The estimated weights of Hg for individuals with the *GSTP1* Val/Val, Ile/Val and Ile/Ile genotypes from the interactive model were 0.25, 0.14, and 0.01, respectively. There was a strong contribution from Hg (0.42) to the negative mixture in association with ASD in the additive gWQS model, but the weight of Hg was considered minimal (0.01) for the *GSTP1* Ile/Ile genotype group, where we found a strong contribution of Hg in the positive gWQS models. Both the positive and negative models suggested that only children with the *GSTP1* Ile/Ile genotype had a strong association of higher Hg concentrations with ASD status. Similarly, we found a strong association between higher Pb concentrations and ASD status only for children with the *GSTP1* Val/Val genotype based on both positive and negative interactive gWQS models. Since the contribution of certain metals (e.g., Cd, Mn for Ile/Val, Pb for Ile/Val) was not strong in either the positive or negative gWQS models, we may conclude that there is no strong association with ASD status.

In order to assess the statistical significance of interactions between blood concentrations of a mixture of metals and each of the GST genes (*GSTP1*, *GSTT1*, *GSTM1*) in relation to ASD status, we compared models with and without interactions, by calculating a test statistic and the corresponding *P*-values following the method reported in Lee *et al.* (Lee et al., 2019)). As shown in Table 4, the interactive associations of *GSTP1* with Hg, Pb, and Mn, based on the positive WQS analysis were not statistically significant for both the unadjusted ($P = 0.97$) and the adjusted models ($P = 0.91$). Based on negative gWQS

analysis, the interactive association between *GSTP1* and these three metals was found to be marginally significant ($P = 0.07$) in the unadjusted model and this interactive association was no longer significant after adjusting for maternal age, parental education levels, parish at child's birth, and consumption of seafood, ($P = 0.24$). These results for the statistical significance of interactions should be interpreted based on both positive and negative WQS models; one should not conclude that there is no significant interactive association only because of non-significant interactions found in the positive model. We provide an additional explanation for this in the following paragraph.

Although our main focus in this paper is to identify potential additive and/or interactive associations of the six metals and each of the three GST genes in relation to ASD status, we also evaluated the *overall* mixture association of six metals based on additive positive and negative gWQS models. In addition to the weights for each metal, in Table 4 we reported the unadjusted and adjusted MORs for the overall mixture index and their corresponding *P*-values based on each of the positive and negative gWQS models. The results from the additive negative gWQS models that assessed the negative association of the overall mixture index score with ASD were statistically significant [MOR (95% CI) = 0.56 (0.42, 0.74); $P < 0.01$] and remained significant in the adjusted model [MOR (95% CI) = 0.70 (0.49, 0.99); $P < 0.05$]. These findings are explained by the weights being spread out over six metals in the negative gWQS models, while the majority of total weights were contributed by a certain set of metals (e.g., As, Al, and Mn) when the positive gWQS was modeled. This can also be the reason why the interactive associations we observed in the positive gWQS models were not statistically significant, while the interactive associations were marginally significant from the unadjusted negative gWQS model. Thus, not surprisingly, the positive WQS model suggests that the association of higher overall index score with ASD status was not statistically significant for both the unadjusted [MOR (95% CI) = 1.08 (0.81, 1.43); $P = 0.60$] and adjusted models MOR (95% CI) = 1.15 (0.74, 1.79); $P = 0.53$]. Similar findings were observed from the interactive gWQS models; the adjusted association between overall mixture index score and ASD status was not statistically significant in the positive model [MOR (95% CI) = 1.20 (0.65, 2.21); $P = 0.56$)], but statistically significant in the negative model [MOR (95% CI) = 0.46 (0.25, 0.84); $P = 0.01$]. We emphasize the need for a clear and precise understanding of these findings of overall associations; the assessment of *overall* mixture association should be carefully addressed especially when the main focus of the study is on assessing *individual* relative associations. Considering that the weight assigned to Pb was almost zero for the *GSTP1* Val/Val genotype from the negative interactive gWQS models, a significant overall mixture association from those models should not be interpreted as all six metals across the genotypes were negatively associated with ASD. In other words, a non-significant overall mixture association from the positive gWQS models does not mean that none of the six metals in the mixture has a significant positive association with ASD.

Similar analyses were conducted for exploring possible interactions between each of the six metals and *GSTM1* as well as *GSTT1* in relation to ASD status. In both positive and negative gWQS models, none of the unadjusted and adjusted interactive associations between metals and *GSTM1* or *GSTT1* genotypes in relation to ASD status were statistically significant. Specifically, in the adjusted positive interactive gWQS model for *GSTM1,* the

contributions of As and Al were 0.38 and 0.20, respectively. The contributions of Mn for children with *GSTM1* genotype (I*) was 0.22. In the adjusted negative interactive gWQS model the contribution of Cd was 0.14 and the contributions of Pb for children with *GSTM1* DD and I* genotypes were 0.25 and 0.40, respectively. The adjusted association between overall mixture index score and ASD status was not statistically significant in the positive model [MOR (95% CI) = 1.27 (0.74, 2.06); $P = 0.34$)], but statistically significant in the negative model [MOR (95% CI) = 0.68 (0.48, 0.98); $P = 0.04$]. Additional details are provided in supplementary Table S.5.

In addition, in the adjusted positive interactive gWQS models for *GSTT1* the contributions of As and Al were 0.42 and 0.26, respectively, and the contribution of Hg for children with *GSTT1* genotype (DD) was 0.15. In the adjusted negative interactive gWQS model the contributions of Cd was 0.13, the contribution of Hg for children with *GSTT1* genotype (I*) was 0.17, and the contributions of Pb for children with *GSTT1* genotypes (DD) and (I*) were 0.14 and 0.13, respectively, The adjusted association between overall mixture index score and ASD status was not statistically significant in the positive model [MOR (95% CI) = 1.17 (0.74, 1.85); $P = 0.51$)], but statistically significant in the negative model [MOR (95% CI) = 0.62 (0.42, 0.92); $P = 0.02$]. Additional details are provided in supplementary Table S.6.

In order to compare our interactive gWQS model results with the CLR results, we fitted a multivariable CLR with the same independent variables that were included in the final interactive gWQS model. In a co-dominant model for *GSTP1*, the interactions between *GSTP1* and blood concentrations of Mn and Pb were not statistically significant. Specifically, after adjusting for As, Cd, Al, and interactions between *GSTP1* and Pb (Pb **GSTP1*), as well as Hg (Hg **GSTP1*), the interaction between *GSTP1* and blood concentrations of Mn was not significant ($P = 0.25$). Similarly, the interaction between *GSTP1* and blood concentrations of Pb while controlling for other metals and the interactions with *GSTP1* was not significant ($P = 0.64$). However, for Hg this interaction was significant ($P = 0.04$). While based on a negative association gWQS model the interactive effect of *GSTP1* with a mixture of Mn, Pb, and Hg was found to be marginally significant ($P = 0.07$) as shown in Table 4, and a model comparison between the interactive model and an additive model with all six metals in terms of −2 log likelihood shown in Table 5 revealed no statistically significant difference, ($P = 0.15$). However, a similar comparison of the results, when adjusted for maternal age, parental education levels, parish at child's birth, and consumption of seafood, in a negative association gWQS and the adjusted interactive CLR models revealed no significant difference between these two models ($P = 0.24$, $P = 0.35$, respectively). Additional details regarding these findings are provided in Table 5. Since As and Cd had high percentage (i.e. >35%) of concentrations below their respective LoDs, we conducted a sensitivity analysis in the process of variable selection for the final model by including and removing As or Cd or both from the final multivariable CLR model. The findings from our sensitivity analysis indicated not significant changes (unadjusted $P = 0.15$, adjusted $P = 0.35$) in the main interactive findings from these models. (Tables S.9–S.11) Therefore, in the final CLR model we keep both As and Cd as additive terms (i.e., no interactions for As and Cd with GST genes) in our final CLR model.

In both CLR and gWQS models, the interactive associations of either *GSTM1* or *GSTT1* with the six metals (without and within a mixture) were not statistically significant. Additional details are provided in supplementary Tables S.7 and S.8.

5. Discussion

5.1 Summary of findings from mixture and individual analysis of metals in relation to ASD

Overall, in this study building on prior methodological work by our group (Lee et al., 2019), in both unadjusted and adjusted negative additive gWQS regression models we found inverse associations of the overall mixture gWQS index score of the six metals (Pb, Hg, Mn, As, Al, Cd) with ASD [MOR (95% CI) = 0.56 (0.24, 0.74); $P < 0.01$) and [MOR (95%CI) = 0.70; (0.49, 0.99); $P < 0.05$], respectively. Also, though in the unadjusted negative gWQS regression model we found a marginally significant interaction between *GSTP1* and three metals (Pb, Hg, and Mn) in a mixture in relation to ASD status (P = 0.07), when adjusted for child's parish at birth, parental education, maternal age, and seafood consumption this interaction was no longer statistically significant (P = 0.24).

Findings from univariable GLMs for Pb and Mn, and from random effect univariable quantile regression models for Hg, Al, As, and Cd that accounted for possible clustering effect caused by the matched pairs, revealed only significantly lower geometric mean Pb concentrations in the ASD cases than in the TD control group, (1.92 vs. 2.34 μg/dL; $P < 0.01$), and a significantly lower median blood Hg concentration in ASD cases, (0.64 vs. 0.81μ/L; $P = 0.01$). However, when adjusted for potential confounding variables that we reported for each of these six metals in the footnote of Table 2, these differences were no longer statistically significant. Furthermore, there was a significant interaction between *GSTP1* and Mn in relation to ASD (P = 0.03) using our interactive CLR dominant models which is consistent with our previously reported finding (Rahbar et al., 2015b). In addition, though from the CLR results we found a significant (P = 0.04) overall interaction between *GSTT1* and blood concentrations of Al in relation to ASD, none of the two genotype-specific P values for this association were significant (P = 0.09 and $P = 0.29$ for DD and I*, respectively).

5.2 Inverse association of blood concentrations for some metals with ASD

A possible explanation for the inverse associations of Pb and Hg with ASD from univariable GLM and quantile regression models as well as the inverse association of the WQS mixture index score with ASD is that some children with ASD eat a more restricted diet compared to TD children, possibly due to the higher rate of comorbid GI issues and sensory sensitivities. For example, we have reported earlier that, compared to TD children, a lower percent of children with ASD eat fish. Since Hg is found in fish, this difference may result in TD children having higher concentrations of Hg compared to the ASD children in univariable analysis. However, when adjusted for the frequency of fish consumption, the difference in the blood Hg concentrations between the ASD and TD control groups was no longer significant statistically (Rahbar et al., 2013).

5.3 Temporality and causality of associations reported in relation to ASD

Since the study design for our study is matched case-control and the measurement of exposure to the six metals took place at the time when the children were evaluated to confirm their ASD status at age 2–8 years, we are unable to establish temporality of the associations reported in this manuscript. Also, we emphasize that the associations reported here may not be causal and reverse causality could be a possible explanation, but the direction of causality may be difficult to establish from our currently available data. For example, as reported earlier, lower concentrations of blood Hg in children with ASD could be associated with a lower consumption of fish and seafood by children with ASD compared to that of TD children, possibly due to a higher prevalence of GI issues and sensory sensitivities in children with ASD compared to that of TD children. On the other hand, lower consumption of fish (Abedi & Sahari, 2014) and seafood (Martins, Bandarra, & Figueiredo-Braga, 2020) could lead to lower levels of omega-3 or higher omega-6 fatty acids in children with ASD that are shown to be important for brain development (Mazahery et al., 2017). Similarly, previous studies in the US reported a significant correlation between levels of Pb in the soil and root vegetables (i.e. carrots, onions, and radishes) ($r = 0.56$, $P < 0.001$) and fruits that were grown in the contaminated soil (Finster, Gray, & Binns, 2004). Since ASD cases may consume fewer fruits and vegetables compared to TD controls, Pb concentrations in blood of ASD children is expected to be lower than that of TD control children in univariable analysis. In the present study, the difference between the adjusted mean concentrations of Pb and Hg between ASD cases and TD controls became smaller and was no longer statistically significant after adjusting for dietary exposures.

5.4 Assessing association of metal concentrations with ASD with a large portion below LoD

Since a sizeable portion of the concentrations for Al, As, and Cd were below their respective LoDs, we compared ASD cases and TD control groups with respect to the median concentrations for Al, As, and the 75th percentile for Cd, but we did not find any significant differences for any of these three metals from both unadjusted and adjusted quantile regression models. These findings are consistent with our previous findings that compared the mean concentration for these three metals from a smaller sample (Rahbar et al., 2016; Rahbar et al., 2012; Rahbar et al., 2014a).

5.5 Interaction between each GST gene and each of the six metals in relation to ASD

We found a significant interaction between *GSTP1* and Mn, which is consistent with our previously reported possible interactive effect of Mn and *GSTP1* in relation to ASD (Rahbar et al., 2015b). In addition, we have demonstrated that the interaction between Mn and *GSTP1* in relation to ASD remained significant in a CLR model with ASD status as the dependent variable that included Mn, *GSTP1*, Mn**GSTP1*, and an estimated mixture score (or mixture index) of four other metals (Pb, Hg, As, and Cd) generated from WQS regression models. However, this mixture analysis did not include Mn in the mixture and controlled for only additive effects of four metals (Pb, Hg, As, and Cd) in the mixture (Rahbar et al., 2018).

5.6 Advantages of mixture analysis using interactive gWQS algorithm developed by Lee *et al*.

Since the pairwise correlations between pairs of metals were generally low to moderate (i.e., ranged from 0.05 to 0.53), we took advantage of mixture analysis that accounts for correlations between metals within a mixture (Czarnota et al., 2015a; Carrico et al., 2015) in relation to ASD. Some studies have used the original version of the WQS regression model to assess additive associations of metal mixtures on the risk of cognitive and neurodevelopmental disorders in children (Sanders, Claus, & Wright, 2015). Sanders et al, also used the original WQS regression model to assess additive associations of environmental exposures to lead, cadmium, mercury, and arsenic in kidney disease among adults without exploring interactions (Sanders et al., 2019). However, one of the main objectives in this study was to investigate the possible interactive associations of each of the GST genes (*GSTP1, GSTT1*, and *GSTM1*) and each of six metals (Pb, Hg, As, Cd, Mn, and Al) in relation to ASD in Jamaican children. For this reason, we explored potential interactions of each of the metals with each of three GST genes under the framework of mixture analysis using the interactive gWQS algorithm that was developed by Lee *et al*. (Lee et al., 2019) in order to (a) account for the potential clustering effect of matched pairs of ASD cases and TD controls and (b) identify interactions between each of the metals within a mixture and each of the GST genes, which cannot be performed by using the original WQS method (Carrico et al., 2015; Czarnota et al., 2015; Gennings et al., 2013; Czarnota et al., 2015b). To the best of our knowledge there are no other published epidemiologic studies that have utilized this interactive gWQS algorithm (Lee et al., 2019) to evaluate whether contributions of the individual metals to their mixture vary by levels of independent variables such as the GST genes. Specifically, in this study we estimated a weighted index in which the stratum-specific weights of metals are determined while accounting for correlation among multiple metals and potential clustering due to data coming from an age-, and sex-matched study as well as censoring of blood metal concentrations due to being below the LoDs.

Although there are some differences, the mixture analysis and analysis of data for each metal individually, using CLR, resulted in somewhat similar findings, indicating potential interaction between blood Mn concentrations and *GSTP1*. In this paper, the findings from the interactive gWQS models suggested that the amount of contribution of Mn, Hg and Pb to the mixture effects on ASD status differed by *GSTP1* genotypes. While these potential interaction effects between each of these metals and *GSTP1* cannot be captured in *additive* gWQS models, both positive and negative *interactive* gWQS models suggested that only children with the *GSTP1* Ile/Ile genotype may have a strong association of higher Mn or Hg concentrations with ASD status, and only children with the *GSTP1* Val/Val genotype may have a strong association between higher Pb concentrations and ASD status. In particular, the finding of potential interaction between Mn and *GSTP1* is consistent with our previous findings from the ERAJ study when an interactive association of blood Mn concentrations and *GSTP1* was initially found by analyzing the role of Mn individually in relation to ASD using CLR. (Rahbar et al., 2015b) The potential interactive associations of some other metals such as Pb and Hg that we found with *GSTP1* based on a mixture analysis were not detected in the models that evaluated the interaction between *GSTP1* and each metal

individually. The findings from mixture analysis in this study also includes interactions between a metal within a mixture of metals and *GSTT1* and *GSTM1* in relation to ASD, which were not statistically significant at a 5% level of significance.

On the other hand, when we fitted a multivariable CLR with the same independent variables that were in the final interactive gWQS model (As, Al, and Cd with additive effects and Mn, Pb, and Hg and their interactions with *GSTP1*), in a co-dominant model for *GSTP1*, the interactions between *GSTP1* and blood concentrations of Mn, Hg, and Pb were not statistically significant. However, in a negative interactive gWQS model the overall interaction effect of *GSTP1* with a mixture of Mn, Pb, and Hg was marginally significant ($P = 0.07$), suggesting that the interactive CLR model shown in Table 5 may not be a significant improvement over the additive model that included only six metals without interactions. Similar comparison of the results from the adjusted (adjusted for the four covariates shown in the footnote of Table 5) negative effect gWQS and the adjusted interactive CLR models revealed no significant difference between these two models. We speculate that the differences between the findings from the gWQS models and the CLR are partly due to the low-moderate level of multicollinearity potentially caused by simultaneous inclusion of the six metals and interaction of *GSTP1* with Mn, Pb, and Hg. However, the gWQS has an internal structure to create a mixture of correlated metals first and then to explore interaction between *GSTP1* and each metal within the mixture, minimizing the effect of multicollinearity in the gWQS models. In addition, all the underlying assumptions of gWQS including that the associations between the concentrations of each metal and ASD should be monotonic, and linear in the quantiles were met.

As mentioned earlier, our findings from the unadjusted and adjusted negative additive gWQS model also revealed inverse associations of the overall mixture index score with ASD. These inverse associations suggest that the ASD group has a significantly lower additive mixture score than the TD control group. These findings are not surprising as we did observe that the contributions of Pb and Hg to the mixture score in the related gWQS model is 42% (weight =0.42 for Pb and Hg) for each of these two metals as shown in Table 4, and Pb and Hg concentrations were significantly lower in the ASD group, compared to the TD control group as shown in Table 1.

We used the gWQS method to address various issues not only for analyzing correlated/clustered data, but also for handling censoring issues due to having concentrations below their respective LoDs for Cd, As, Al, and Hg. As shown by Lee *et al.* (Lee et al., 2019), the benefits of gWQS include its robustness to censored data, because it is uses quantile scores, rather than actual concentrations of metals. Since the gWQS regression is constrained to model associations between the outcome and exposure variables (e.g., blood metal concentrations) in one direction, in order to properly estimate both magnitude and direction of the individual exposure effects, we built a model with each direction for the weighted index (i.e., positive and negative). If the contribution of a certain metal exposure is not considered adequate for both positive and negative models, we may conclude that there is no significant association of the exposure with an outcome such as ASD status in the presence of other exposures in the mixture. To reduce the complexity of the gWQS estimation process that may arise when interactions are evaluated for multiple exposures and multiple

covariates, we suggest exploring interactions for one exposure at a time, before conducting the interactive gWQS analysis (Lee et al., 2019) for all exposures together.

6. Limitations

We acknowledge some limitations in this study. First, though we have used quantile regression to minimize the potential bias in estimation of median blood concentrations for Hg, Al, and As, as well as the 75th percentile for Cd blood concentrations, we acknowledge that replacement of concentrations below the LoD (left censored data) by simple substitution such as (LoD/√2) may still cause some bias in estimation of medians, as well as the 75th percentiles and their respective standard deviations. This bias is also influenced by the percentage of measurements below LoD and the overall sample size used (Ganser & Hewett, 2010; Hewett & Ganser, 2007). Since the percentage below LoD for Hg, Al, As and Cd ranged from16.7% – 55.5%) and we have used a relatively large sample size (n=532 children) in quantile regression models for estimation of median concentrations for Hg, Al, and As and the 75th percentile for Cd, we believe that the bias in our estimation procedures should be reasonably close to minimum. Nevertheless, for dichotomizing As and Cd as (> LoD vs. ≤LoD), we have conducted sensitivity analyses to assess and report the impact of keeping or removing these binary As and Cd variables as additive terms in the multivariable CLR model.

The assessment of overall mixture association should be carefully addressed for both the positive and negative models when the main focus of the study is to assess individual relative associations of exposures and their potential interactive associations with other variables in relation to an outcome variable such as ASD (Lee et al., 2019; Kordas et al., 2018). We acknowledge that this study is not only focused on testing for an overall mixture association of multiple metals, but also on estimating stratum-specific weights for individual metals that may differ by genotype for the three GST genes using the interactive gWQS algorithm (Lee et al., 2019). The relative strength of stratum-specific individual exposures within a mixture, which is determined by estimated weights, may not be fully evaluated by the overall association estimate that is likely to be determined by the association of the majority of the exposures. Specifically, a non-significant overall mixture association from the positive interactive gWQS models should not be interpreted as showing that none of the six metals in the mixture has a significant positive association with ASD status. For additional discussion regarding the model assumptions of the interactive gWQS model we would refer readers to the Lee *et al.* gWQS methods paper (Lee et al., 2019).

We have also shown that children with ASD have significantly different diet and food consumption when compared with TD children. These findings could partly explain the observation of lower blood metal concentrations of Pb and Hg in children with ASD compared to TD control children. When analyzing the association of individual metals with ASD status, one should identify potential confounding variables, including dietary factors. However, identification of dietary factors as potential confounders may be more challenging for the mixture index.

7. Conclusions

Findings from the unadjusted additive gWQS mixture analysis of the six metals in relation to ASD are somewhat similar to our previously reported findings that were confirmed by reassessing the same associations based on analysis of the role of individual metals in additive GLM for Pb and Mn and quantile regression at the median concentrations for Hg, Al, and As, and at the 75th percentile for Cd. However, we found some differences in the findings from the interactive gWQS and CLR models that investigated the same interactions between a specific GST gene and a mixture of the same metals. Specifically, though we found a marginally significant association between *GSTP1* and a mixture of Mn, Pb, and Hg in relation to ASD, in the adjusted model this interaction was no longer significant. The finding of a potential role of *GSTP1* as an effect modifier when assessing the relationship of blood Mn concentrations and ASD based on the unadjusted gWQS model is consistent with our previous reports. However, in the multivariable interactive CLR this interaction was not statistically significant. We believe that the difference between these two findings, is partly due to a low level of multicollinearity in the CLR caused by low-moderate level of correlation between the concentrations of the six metals. In contrast, the mixture analysis first accounts for correlations between metals by developing a mixture of metals and also provides useful information about the positive and negative effects of the mixture index on ASD for both additive and interactive models. The findings from this study also emphasize the importance of exploring potential interactions between each of the metals and each of the genes in a proper way while accounting for complex data correlations and structures among multiple metals when their associations with ASD status are assessed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Humans are exposed to mixtures of metals and other environmental chemicals
- We used mixture analysis to assess relative contribution of six metals in ASD
- We found a possible effect modification of *GSTP1* with Mn, Pb, and Hg in ASD
- Our finding of interaction of *GSTP1* with Mn in ASD has been reported previously
- Possible effect modification of *GSTP1* with Pb, and Hg in ASD requires replication

Table 1.

Characteristics of children and their parents by ASD case status (266 matched pairs, n = 532 children)

Variables	Categories	ASD case (n=266) N (%)	TD control (n=266) N (%)	Matched OR (95% CI)	*P*-Value*
Child's sex	Male	217 (81.6)	217 (81.6)	N/A	1.00
Child's age (months)	Age < 72 Age ≥ 72	185 (69.5) 81 (30.5)	190 (71.4) 76 (28.6)	0.44 (0.14, 1.44)	0.18
Child's race	Afro-Caribbean	254 (95.5)	258 (97.0)	1.50 (0.61, 3.67)	0.37
Maternal age *a* (at child's birth)	Age < 35 Age ≥ 35	215 (81.1) 50 (18.9)	229 (87.7) 32 (12.3)	0.58 (0.36, 0.94)	0.03
Paternal age *b* (at child's birth)	Age < 35 Age ≥ 35	141 (54.0) 120 (46.0)	176 (69.0) 79 (31.0)	0.48 (0.32, 0.71)	<0.01
Maternal race	Afro-Caribbean	256 (96.2)	259 (97.4)	1.43 (0.54, 3.75)	0.47
Paternal race *c*	Afro-Caribbean	254 (95.9)	255 (97.0)	1.38 (0.55, 3.42)	0.49
Maternal education *d* (at child's birth)	Up to high school † Beyond high school ††	137 (51.5) 129 (48.5)	164 (62.4) 99 (37.6)	0.69 (0.50, 0.95)	0.02
Paternal education *e* (at child's birth)	Up to high school † Beyond high school ††	150 (58.6) 106 (41.4)	195 (77.7) 56 (22.3)	0.44 (0.29, 0.65)	<0.01
Socioeconomic status (SES)	Car ownership	150 (56.4)	106 (39.9)	0.50 (0.35, 0.72)	<0.01
GSTP1 *f*	Ile/Ile Ile/Val Val/Val	68 (25.9) 144 (54.8) 51 (19.4)	65 (24.4) 139 (52.3) 62 (23.3)	Reference 1.03 (0.67, 1.57) 1.30 (0.76, 2.22)	0.58
GSTM1 *g*	DD *i* I/I or I/D *j*	78 (29.7) 185 (70.3)	62 (23.6) 201 (76.4)	1.37 (0.93, 2.03)	0.11
GSTT1 *h*	DD *i* I/I or I/D *j*	70 (26.6) 193 (73.4)	65 (24.8) 197 (75.2)	1.08 (0.74, 1.59)	0.70
Lead (Pb) (μg/dL)	Geometric mean *k*	1.92	2.34	-	<0.01 **
Mercury (Hg) (μg/L)	Median *l*	0.64	0.81	-	0.01 ***
Arsenic (As) (μg/L)	Median *l*	2.24	2.29	-	0.55 ***
Cadmium (Cd) (μg/L)	75th percentile *m*	0.20	0.20	-	1.00 ***
Aluminum (Al) (μg/L) *n*	Median *l*	10.00	9.74	-	0.86 ***
Manganese (Mn) (μg/L)	Arithmetic mean *o*	10.29	10.30	-	0.95 **

* *P*-Values are based on Wald's test in conditional logistic regression models.

** We used univariable General Linear Models (GLM) and adjusted for pairs.

*** We used univariable quantile regression models and adjusted for pairs.

† Up to high school education means attended Primary/Jr. Secondary, and Secondary/High/Technical schools.

†† Beyond high school education means attended a Vocational, Tertiary College, or University.

[a] Maternal age was missing for 1 ASD case and 5 TD controls.

[b] Paternal age was missing for 5 ASD cases and 11 TD controls.

[c] Paternal race was missing for 1 ASD case and 3 TD controls.

[d] Maternal education was missing for 3 TD controls.

[e] Paternal education was missing for 10 ASD cases and 15 TD controls.

[f] *GSTP1* genotype was missing for 3 ASD cases.

[g] *GSTM1* genotype was missing for 3 ASD cases and 3 TD controls.

[h] *GSTT1* missing for 3 ASD cases and 4 TD controls.

[i] DD indicates the null alleles for *GSTT1* and *GSTM1*.

[j] I/I or I/D indicate the homozygote (I/I) or a heterozygote (I/D) for *GSTT1 and GSTM1*.

[k] Since the distribution of blood lead (Pb) concentrations were not normal for both ASD cases and TD controls, we reported geometric mean (Exp. [Mean (ln Pb concentration)]).

[l] Median estimates are based on quantile regression.

[m] We used quantile regression analysis at 75th percentile.

[n] One concentration was missing for Al.

[o] Since the distribution of blood manganese (Mn) concentrations was normal for both ASD cases and TD controls, we reported arithmetic means for Mn.

Table 2.

Multivariable associations of metal concentrations with ASD status based on General Linear Models for Pb and Mn and Quantile Regression Models for Hg, As, Al, and Cd. [n = 532 children (266 matched pairs)]

Metals	Adjusted [f]				
	n	% below LoD	ASD cases Mean*/Media n**	TD controls Mean*/Median**	*P*-Value
Pb (μg/dL) [a]	507	0.0	2.21	2.30	0.53 *
Hg (μg/L) [b]	509	16.7	0.48 [e]	0.55 [f]	0.30 **
As (μg/L) [b]	508	38.9	1.96 [e]	1.66 [f]	0.40 **
Cd (μg/L) [c]	513	55.5	0.20	0.20	0.67
Al (μg/L) [b]	513	29.8	14.99 [e]	13.85 [f]	0.44 **
Mn (μg/L) [d]	513	0.0	10.23	10.52	0.41 *

* We used univariable General Linear Models (GLM) and adjusted for potential correlation between the measurements for cases and control groups due to matched pairs.

** We used univariable quantile regression models and adjusted for potential correlation between the measurements for cases and control groups due to matched pairs.

[a] Since the distribution of blood lead (Pb) concentrations was not normal for both ASD cases and TD controls, we reported geometric mean (Exp. [Mean (ln Pb concentration)])

[b] For mercury (Hg), arsenic (As), and aluminum (Al) we reported median.

[c] We used quantile regression analysis at the 75th percentile of blood concentration for cadmium (Cd).

[d] Since the distribution of blood manganese (Mn) concentrations was normal for both ASD cases and TD controls, we reported arithmetic means for Mn.

[e] We used quantile regression model at median for Hg, As, and Al.

[f] Potential confounders that we used for adjustment in analysis for each of the metals were selected from those reported in our previous papers as shown below:

- **For Pb:** maternal age, parental education levels, parish at child's birth, SES (i.e., car ownership by the family), consumption of shellfish (lobsters, crabs), and teflon (pots, pans, and dishes used for cooking).
- **For Hg:** frequency of seafood consumption, maternal age, and parental education.
- **For As:** car ownership, maternal age, parental education levels, source of drinking water, consumption of "yam, sweet potato, or dasheen", "carrot or pumpkin", "callaloo, broccoli, or pak choi", cabbage, and the frequency of seafood consumption per week.
- **For Cd:** parental education levels, parish of child's birth (in "Kingston, St. Andrew, or St. Catherine parishes vs. other parishes), consumption of seafood (more than 6 meals per week), "yam, sweet potato, or dasheen", cabbage, fried plantain, boiled dumpling, and white bread.
- **For Al:** parental education levels, consumption of root vegetables (yam, sweet potato, or dasheen), avocado, and tuna (canned fish).
- **For Mn:** paternal age, parental education, place of child's birth (Kingston parish), consumption of root vegetables, cabbage, saltwater fish, and cakes/buns.

Table 3.

Association of blood metal concentrations with ASD status among children with different *GSTP1, GSTM1,* and *GSTT1* genotypes based on interactive (metal *GST gene) models using CLR (Conditional Logistic Regression) models.

Metal	Genetic genotype	Genetic Model	Genotype (s)	Dichotomized/Quartiles	Matched OR (95% CI)	*P*-Value	*P*-Value for Interaction
Mn	*GSTP1*	Co-dominant	Ile/Ile	≥75th vs. <75th	1.81 (0.88, 3.70)	0.11	0.09
			Ile/Val	≥75th vs. <75th	0.84 (0.48, 1.48)	0.55	
			Val/Val	≥75th vs. <75th	0.57 (0.22, 1.44)	0.23	
		Dominant	Val/*	≥75th vs. <75th	0.77 (0.47, 1.25)	0.29	0.03
			Ile/Ile	≥75th vs. <75th	1.87 (0.92, 3.38)	0.09	
Pb	*GSTP1*	Co-dominant	Ile/Ile	2 vs 1	0.49 (0.15, 1.51)	0.22	0.75
				3 vs1	0.25 (0.08, 0.76)	<0.01	
				4 vs 1	0.25 (0.08, 0.79)	<0.01	
			Ile/Val	2 vs 1	0.62 (0.32, 1.21)	0.16	
				3 vs1	0.50 (0.25, 0.99)	0.05	
				4 vs 1	0.58 (0.30, 1.14)	0.11	
			Val/Val	2 vs 1	0.57 (0.21, 1.61)	0.29	
				3 vs1	0.21 (0.07, 0.67)	<0.01	
				4 vs 1	0.42 (0.14, 1.27)	0.12	
		Dominant	Val/*	2 vs 1	0.62 (0.35, 1.09)	0.10	0.63
				3 vs1	0.40 (0.22, 0.71)	<0.01	
				4 vs 1	0.53 (0.30, 0.95)	<0.01	
			Ile/Ile	2 vs 1	0.48 (0.16, 1.50)	0.21	
				3 vs1	0.25 (0.09, 0.76)	<0.01	
				4 vs 1	0.25 (0.08, 0.77)	<0.01	
Hg	*GSTP1*	Co-dominant	Ile/Ile	≥LoD vs. <LoD	0.69 (0.27, 1.81)	0.45	0.12
			Ile/Val	≥LoD vs. <LoD	0.21 (0.10, 0.45)	<0.01	
			Val/Val	≥LoD vs. <LoD	0.58 (0.21, 1.59)	0.29	
		Dominant	Val/*	≥LoD vs. <LoD	0.67 (0.27, 1.73)	0.40	0.15
			Ile/Ile	≥LoD vs. <LoD	0.30 (0.17, 0.53)	<0.01	
Al	*GSTP1*	Co-dominant	Ile/Ile	≥LoD vs. <LoD	0.54 (0.24, 1.20)	0.13	0.17
			Ile/Val	≥LoD vs. <LoD	1.13 (0.64, 1.98)	0.68	
			Val/Val	≥LoD vs. <LoD	1.53 (0.66, 3.54)	0.32	
		Dominant	Val/*	≥LoD vs. <LoD	1.25 (0.77, 2.01)	0.37	0.08
			Ile/Ile	≥LoD vs. <LoD	0.57 (0.26, 1.26)	0.16	
Pb	*GSTM1*		DD	2 vs 1	0.68 (0.27, 1.70)	0.41	0.49
				3 vs1	0.30 (0.11, 0.84)	0.02	
				4 vs 1	0.29 (0.11, 0.75)	<0.01	
			I*	2 vs 1	0.47 (0.26, 0.88)	<0.01	

Metal	Genetic genotype	Genetic Model	Genotype (s)	Dichotomized/Quartiles	Matched OR (95% CI)	*P*-Value	*P*-Value for Interaction
				3 vs1	0.38 (0.21, 0.70)	<0.01	
				4 vs 1	0.50 (0.27, 0.95)	0.03	
Hg	***GSTM1***		DD	≥LoD vs. <LoD	0.51 (0.34, 1.72)	0.18	0.40
			I*	≥LoD vs. <LoD	0.30 (0.17, 0.56)	<0.01	
Mn	***GSTM1***		DD	≥75th vs. <75th	0.94 (0.42, 2.09)	0.88	0.68
			I*	≥75th vs. <75th	1.13 (0.71, 1.81)	0.60	
Pb	***GSTT1***		DD	2 vs 1	0.62 (0.23, 1.66)	0.34	0.82
				3 vs1	0.27 (0.09, 0.80)	<0.01	
				4 vs 1	0.46 (0.18, 1.21)	0.12	
			I*	2 vs 1	0.51 (0.28, 0.93)	0.03	
				3 vs1	0.41 (0.22, 0.73)	<0.01	
				4 vs 1	0.42 (0.22, 0.78)	<0.01	
Al	***GSTT1***		DD	≥LoD vs. <LoD	2.00 (0.90, 4.47)	0.09	0.04
			I*	≥LoD vs. <LoD	0.77 (0.48, 1.25)	0.29	
Hg	***GSTT1***		DD	≥LoD vs. <LoD	0.29 (0.11, 0.76)	<0.01	0.58
			I*	≥LoD vs. <LoD	0.39 (0.22, 0.70)	<0.01	
As	***GSTT1***		DD	≥LoD vs. <LoD	1.18 (0.54, 2.57)	0.67	0.07
			I*	≥LoD vs. <LoD	0.56 (0.33, 0.96)	0.03	
Mn	***GSTT1***		DD	≥75th vs. <75th	1.67 (0.77, 3.59)	0.19	0.13
			I*	≥75th vs. <75th	0.84 (0.51, 1.37)	0.48	

For *GSTP1,* n = 524 children (262 matched pairs); For *GSTM1,* n = 518 children (259 matched pairs); For *GSTT1,* n= 516 children (258 matched pairs).

Mn was categorized into binary (≥75th vs. <75th), Pb was categorized into quartiles, and Hg, As, Al, and Cd were categorized into binary (≥limit of detection (LoD) vs. <LoD).

Table 4.

Additive (six metals) and interactive (metals * *GSTP1*) associations with ASD status based on negative and positive association gWQS Models, (n = 240 matched pairs, n = 480 children)

Model		**Positive association gWQS Model**		**Negative association gWQS Model**	
		Unadjusted	**Adjusted***	**Unadjusted**	**Adjusted***
	Metals in gWQS	**Weight**	**Weight**	**Weight**	**Weight**
Additive (six metals)	Lead (μg/dL)	0.000003	0.03	0.42	0.24
	Mercury (μg/L)	0.000003	0.001	0.42	0.45
	Arsenic (pg/L)	0.07	0.46	0.03	0.02
	Cadmium (pg/L)	0.02	0.03	0.09	0.17
	Aluminum (pg/L)	0.39	0.22	0.03	0.08
	Manganese (pg/L)	0.52	0.26	0.02	0.04
	Overall association, MOR of gWQS index (95% CI)	1.08 (0.81, 1.43)	1.15 (0.74, 1.79)	0.56 (0.42, 0.74)	0.70 (0.49, 0.99)
	***P*-Value**	0.60	0.53	<0.01	<0.05
Interactive (metals with *GSTP1*)	**Metals in gWQS**	**Weight**	**Weight**	**Weight**	**Weight**
	Arsenic (μg/L)	0.04	0.27	0.02	0.01
	Cadmium (μg/L)	0.01	0.02	0.04	0.07
	Aluminum (μg/L)	0.28	0.14	0.02	0.04
	Manganese, *GSTP1* (Ile/Ile)	0.15	0.17	0.02	0.02
	Manganese, *GSTP1* (Ile/Val)	0.09	0.02	0.03	0.08
	Manganese, *GSTP1* (Val/Val)	0.02	0.04	0.19	0.19
	Mercury, *GSTP1* (Ile/Ile)	0.02	0.15	0.01	0.01
	Mercury, *GSTP1* (Ile/Val)	0.01	0.05	0.14	0.14
	Mercury, *GSTP1* (Val/Val)	0.0007	0.001	0.25	0.27
	Lead, *GSTP1* (Ile/Ile)	0.000001	0.001	0.18	0.09
	Lead, *GSTP1* (Ile/Val)	0.0004	0.0004	0.09	0.08
	Lead, *GSTP1* (Val/Val)	0.37	0.13	0.0004	0.001
	MOR of gWQS index (95% CI)	1.07 (0.76, 1.51)	1.20 (0.65, 2.21)	0.33 (0.20, 0.54)	0.46 (0.25, 0.84)
	***P*-Value**	0.71	0.56	<0.01	0.01
Model comparison (overall interactive association)	***P*-Value**	0.97	0.91	0.07	0.24

In all calculations we used log of blood metal concentrations, except for manganese (Mn)

*Adjusted for maternal age (<35 vs. ≥35 years), parental education levels (both parents had education up to high school vs. at least one of the parents had education beyond high school), parish at child's birth (Kingston parish vs. other parishes), and consumption of seafood (≤ 6 meals per week vs. > 6 meals per week) as potential confounders or covariates in the models.

Table 5.

Association of six metals [three metals with additive associations (As, Cd, and Al) and the other three with interactive associations (metals**GSTP1)]* in ASD status from multivariable CLRs (Conditional Logistic Regressions), (240 matched pairs, n = 480 children)

Metal, *GSTP1* (genotype)	Adjusted only for the other metals and *GSTP1*			Fully adjusted*		
	Matched OR (95% CI)	*P*-Value	*P*-Value for Interaction	Matched OR 95% CI	*P*-Value	*P*-Value for Interaction
Arsenic (μg/L)	0.98 (0.55, 1.71)	0.93	-	1.23 (0.66, 2.29)	0.51	-
Cadmium (μg/L)	1.07 (0.69, 1.66)	0.77	-	0.98 (0.60, 1.60)	0.93	-
Aluminum (μg/L)	0.99 (0.60, 1.62)	0.97	-	1.05 (0.61, 1.80)	0.86	-
Manganese, *GSTP1* (Ile/Ile)	1.53 (0.67, 3.46)	0.31		1.80 (0.73, 4.48)	0.20	
Manganese, *GSTP1* (Ile/Val)	0.78 (0.40, 1.52)	0.46	0.25	0.82 (0.38, 1.74)	0.60	0.34
Manganese, *GSTP1* (Val/Val)	0.55 (0.19, 1.57)	0.27		0.78 (0.25, 2.51)	0.68	
Mercury, *GSTP1* (Ile/Ile)	0.81 (0.26, 2.57)	0.73		0.92 (0.26, 3.31)	0.91	
Mercury, *GSTP1* (Ile/Val)	0.16 (0.06, 0.40)	<0.01	0.04	0.21 (0.08, 0.57)	<0.01	0.16
Mercury, *GSTP1* (Val/Val)	0.74 (0.23, 2.35)	0.61		0.63 (0.18, 2.22)	0.47	
Lead (2 vs 1), *GSTP1* (Ile/Ile)	0.43 (0.13, 1.44)	0.17		0.40 (0.10, 1.53)	0.18	
Lead (3 vs 1), *GSTP1* (Ile/Ile)	0.26 (0.08, 0.87)	0.03		0.31 (0.08, 1.16)	0.08	
Lead (4 vs 1), *GSTP1* (Ile/Ile)	0.26 (0.07, 0.94)	0.04		0.25 (0.06, 1.06)	0.06	
Lead (2 vs 1), *GSTP1* (Ile/Val)	0.76 (0.36, 1.60)	0.48		0.89 (0.40, 2.00)	0.78	
Lead (3 vs 1), *GSTP1* (Ile/Val)	0.73 (0.35, 1.63)	0.47	0.64	1.10 (0.44, 2.56)	0.90	0.52
Lead (4 vs 1), *GSTP1* (Ile/Val)	0.70 (0.33, 1.49)	0.36		1.13 (0.48, 2.56)	0.78	
Lead (2 vs 1), *GSTP1* (Val/Val)	0.79 (0.26, 2.39)	0.67		0.75 (0.22, 2.51)	0.64	
Lead (3 vs 1), *GSTP1* (Val/Val)	0.24 (0.06, 0.97)	0.03		0.28 (0.07, 1.15)	0.08	
Lead (4 vs 1), *GSTP1* (Val/Val)	0.51 (0.16, 1.61)	0.25		0.76 (0.21, 2.67)	0.66	
Model comparison ** **(overall interactive association)**	***P*-Value**	0.15		***P*-Value**	0.35	

* Fully adjusted refers to a CLR model that in addition to adjusting for the other metals and *GSTP1* it also adjusts for maternal age (<35 vs. ≥35 years), parental education levels (both parents had education up to high school vs. at least one of the parents had education beyond high school), parish at child's birth (Kingston parish vs. other parishes), and consumption of seafood (≤ 6 meals per week vs. > 6 meals per week) as potential confounders or covariates in the models.

** Model comparison in the final CLR model for *GSTP1* is for investigating a possible difference between the additive and interactive (metals**GSTP1*) models. Mn was categorized into binary (≥75th vs. <75th), Pb was categorized into quartiles, and Hg, As, Al, and Cd were categorized into binary (≥limit of detection (LoD) vs. <LoD).

- **Under a co-dominant genetic model for *GSTP1*, the CLR model that adjusts only for the other metals and *GSTP1* is characterized by:** logit P (ASD=1) = β1 (As) + β2 (Al) + β3 (Cd) + β4 (*GSTP1* Ile/Val) + β5 (*GSTP1* Val/Val) + β6 (Mn) + β7 (Hg) + β8 (Pb (2 vs 1)) + β9 (Pb (3 vs 1)) + β10 (Pb (4 vs 1)) + β11 (Mn**GSTP1* Ile/Val) + β12 (Mn**GSTP1* Val /Val) + β13 (Hg**GSTP1* Ile/Val) + β14 (Hg**GSTP1* Val/Val) + β15 (Pb (2 vs 1)**GSTP1* Ile/Ile) + β16 (Pb (3 vs 1)**GSTP1* Ile/Ile) + β17 (Pb (4 vs 1)**GSTP1* Ile/Ile) + β18 (Pb (2 vs 1)**GSTP1* Ile/Val) + β19 (Pb (3 vs 1)**GSTP1* Ile/Val) + β20 (Pb (4 vs 1)**GSTP1* Ile/Val) + β21 (Pb (2 vs 1)**GSTP1* Val/Val) + β22 (Pb (3 vs 1)**GSTP1* Val/Val) + β23 (Pb (4 vs 1) **GSTP1* Val/Val)
- **Under a co-dominant genetic model for *GSTP1*, the fully Adjusted CLR model is characterized by:** logit P (ASD=1) = β1 (As) + β2 (Al) + β3 (Cd) + β4 (*GSTP1* Ile/Val) + β5 (*GSTP1* Val/Val) + β6 (Mn) + β7 (Hg) + β8 (Pb (2 vs 1)) + β9 (Pb (3 vs 1)) + β10 (Pb (4 vs 1)) + β11 (Mn**GSTP1* Ile/Val) + β12 (Mn**GSTP1* Val /Val) + β13 (Hg**GSTP1* Ile/Val) + β14 (Hg**GSTP1* Val /Val) + β15 (Pb (2 vs 1)**GSTP1* Ile/Ile) + β16 (Pb (3 vs 1)**GSTP1* Ile/Ile) + β17 (Pb (4 vs 1)**GSTP1* Ile/Ile) + β18 (Pb (2 vs 1)**GSTP1* Ile/Val) + β19 (Pb (3 vs 1)**GSTP1* Ile/Val) + β20 (Pb (4 vs 1)**GSTP1* Ile/Val) + β21 (Pb (2 vs 1)**GSTP1* Val/Val) + β22 (Pb (3 vs

1)*GSTP1 Val/Val) + β23 (Pb (4 vs 1)*GSTP1 Val/Val)+ β24 (maternal age) + β25 (parental education levels) + β26 (parish at child's birth) + β27 (consumption of seafood)