Maternal polymorphisms in glutathione-related genes are associated with maternal mercury concentrations and early child neurodevelopment in a population with a fish-rich diet

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Abstract

Introduction: Glutathione (GSH) pathways play a key role the metabolism and elimination of the neurotoxicant methylmercury (MeHg). We hypothesized that maternal genetic variation linked to GSH pathways could influence MeHg concentrations in pregnant mothers and children and thereby also affect early life development.

Methods: The *GCLM* (rs41303970, C/T), *GCLC* (rs761142, T/G) and *GSTP1* (rs1695, A/G) polymorphisms were genotyped in 1449 mothers in a prospective study of the Seychellois population with a diet rich in fish. Genotypes were analyzed in association with maternal hair and blood Hg, fetal blood Hg (cord blood Hg), as well as children's mental (MDI) and motor development (PDI; MDI and PDI assessed by Bayley Scales of Infant Development at 20 months). We also examined whether genotypes modified the association between Hg exposure and developmental outcomes.

Results: *GCLC* rs761142 TT homozygotes showed statistically higher mean maternal hair Hg (4.12 ppm) than G carriers (AG 3.73 and GG 3.52 ppm) (p=0.037). For the combination of *GCLC* rs761142 and *GCLM* rs41303970, double homozygotes TT+CC showed higher hair Hg (4.40 ppm) than G+T carriers (3.44 ppm; p=0.018). No associations were observed between *GSTP1* rs1695 and maternal hair Hg or between any genotypes and maternal blood Hg or cord blood Hg. The maternal *GSTP1* rs1695 rare allele (G) was associated with a lower MDI among children (β=-1.48, p=0.048). We also observed some interactions: increasing Hg in maternal and cord blood was associated with lower PDI among *GCLC* rs761142 TT carriers; and increasing Hg in hair was associated with lower MDI among *GSTP1* rs1695 GG carriers.

Conclusions: Maternal genetic variation in genes involved in GSH synthesis is statistically associated with Hg concentrations in maternal hair, but not in maternal or fetal blood. We observed interactions that suggest maternal GSH genetics may modify associations between MeHg exposure and neurodevelopmental outcomes.

Keywords: Methylmercury, GCLC, GCLM, GSTP1, neurodevelopment

1 Introduction

Fish is the main source of human low-level methylmercury (MeHg) exposure. At high levels, MeHg has clear detrimental effects on the nervous system (Clarkson et al. 2003), but the neurotoxic effects of low-level exposure are not established. The developing brain is particularly sensitive to neurotoxicants including MeHg (Costa et al. 2004; Johansson et al. 2007), but it is unclear at what MeHg level the fetal brain is affected. Consequently, it is unclear if fish ingestion poses a risk for fetal toxicity. Research results of MeHg exposure from fish consumption in relation to neurodevelopmental outcomes in children have been contradictory between studies of different populations, with adverse associations observed in some studies (Grandjean et al. 1997; Vejrup et al. 2016), but not in others (Daniels et al. 2004; Davidson et al. 1998; Llop et al. 2012; Strain et al. 2015). Several studies have suggested that genetics may contribute to MeHg body burden as well as to defense mechanisms against MeHg toxicity (Andreoli and Sprovieri 2017; Llop et al. 2015).

An important mechanism in MeHg metabolism involves the conjugation of MeHg to the small tripeptide glutathione (GSH), which facilitates elimination of the conjugate in the bile via the ABC-transporter system (Ballatori and Clarkson 1985). The rate-limiting enzyme for GSH synthesis is γ-glutamyl-cysteine ligase (GCL), which is composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM) (Lu 2013). Further, the conjugation of GSH to MeHg has been suggested to be catalyzed by glutathione S-transferases, particularly the pi 1 isoform (GSTP1) (Custodio et al. 2004). Genetic polymorphisms in *GCLC*, *GCLM*, and *GSTP1* have been linked to MeHg retention and body burden in adults (Barcelos et al. 2013; Custodio et al. 2004; Goodrich et al. 2011; Parajuli et al. 2016; Schlawicke Engstrom et al. 2008). In addition, our group has recently shown that GSTP1 polymorphisms, expressed in *Drosophila*, may influence MeHg toxicity during development through both toxicokinetic and toxicodynamic mechanisms (Vorojeikina et al. 2017).

Accordingly, we hypothesized that maternal polymorphisms in the GSH pathway could modify maternal MeHg body burden, and thereby influence MeHg exposure in the fetus and, as a consequence influence early child neurodevelopment. We have genotyped maternal SNPs in *GCLM*, *GCLC* and *GSTP1* in 1449 pregnant women from a population in the Seychelles with a diet rich in fish and in whom no consistent adverse associations between maternal MeHg exposure and neurodevelopment were observed in their children (Strain et al. 2015; Strain et al. 2012; van Wijngaarden et al. 2017). SNPs were analyzed in association with MeHg biomarker concentrations in mothers (hair and blood) and children (cord blood), as

well as early neurodevelopmental outcomes in children (Bayley scales of infant development; BSID). The influence of an interaction between SNPs and biomarkers of MeHg exposure upon neurodevelopment endpoints was also studied, since antioxidative effects of glutathione may be protective against oxidative stress generated by MeHg (Kaur et al. 2006).

2 Materials and methods

2.1 Study population

This prospective cohort consists of mother-child pairs from the Republic of Seychelles in the Indian Ocean and is of mixed African, European and East Asian origin. Participants were recruited for the Seychelles Child Development Study (SCDS) Nutrition Cohort 2 (NC2), a longitudinal observational study with the overall aim to investigate the effects of MeHg exposure from maternal fish consumption during pregnancy, nutritional status, and genetic predisposition on child developmental outcomes. NC2 consists of 1535 apparently healthy mothers recruited between the years 2008 to 2011 during their first antenatal visit (from 14 weeks of gestation) at eight health centers across the main Island Mahé. Inclusion criteria for NC2 included being native Seychellois, being ≥16 y of age, having a singleton pregnancy, and having no obvious health concerns. Further information on recruitment criteria and power calculations for NC2 has previously been described (Strain et al. 2015). Mothers completed a retrospective fish use questionnaire at 28 weeks gestation, to estimate their weekly consumption of fish during pregnancy. Non-fasting blood samples were collected at 28 weeks gestation, and cord blood and maternal hair were collected at delivery. Whole blood samples were processed at the Public Health Laboratory at the Ministry of Health. One aliquot was shipped to the University of Rochester for Hg analysis and a second aliquot was shipped to Lund University for genotyping.

For prenatal biomarker analyses, participants without genetic data and one each of thirty sibling pairs were excluded; also, missing data varied for the three biomarkers. (A flow chart of the participants included in this study is presented in Supplemental Figure S1). DNA from blood for genotyping was available for 1449 mothers. DNA and biomarker values were available for 1311, 1379, and 1004 mother child pairs for hair, maternal blood, and cord blood, respectively. For the BSID endpoints, exclusions were determined as described in Strain et al. (2015) and included pre- or perinatal deaths, maternal pre- or perinatal complications, birthweight<1500g, head trauma, twin births and seizures or disability.

Additionally, participants without genetic data and one each of thirteen sibling pairs were excluded. There were 1330 pairs eligible for models for the BSID endpoints, 1230, 1266, and 935 of whom had samples of hair, maternal and cord blood respectively. The study was conducted according to guidelines laid down in the Declaration of Helsinki and all study procedures involving participants were reviewed and approved by the Seychelles Ethics Board, the Research Subjects Review Board at the University of Rochester, and the Regional Ethics Committee at Lund University, Sweden.

2.2 Hg measurements

Hair samples were cut at delivery and the longest available segment of maternal hair growing during gestation was analyzed assuming a hair growth rate of 1.1 cm/month. Total mercury in maternal hair during gestation is an established biomarker for prenatal MeHg exposure and has been used to monitor neurotoxicity of methylmercury; maternal hair Hg is known to correlate with infant brain Hg levels (Cernichiari, et al. 1995) and is believed to reflect the species of Hg that is transported across the blood-brain barrier (Clarkson & Magos, 2006). Total Hg in hair was measured by cold-vapor atomic-absorption-spectrometry (CVAAS) as previously described (Cernichiari et al. 1995) and reported in parts per million (ppm). Total Hg was measured on stored maternal and cord whole blood samples with atomic fluorescence spectrometry using a PSA Millennium Merlin System (PS Analytical, Kent, UK). The limit of detection for THg in maternal hair was 0.14 ppm and our limit of detection for Hg in blood was approximately 0.01 ng/mL, depending on sample volume (Pichichero et al. 2008).

2.3 Neurodevelopmental assessment

Toddlers completed developmental testing with the Bayley Scales of Infant Development (BSID-II) at 20 months (range: 15-32 months). The BSID-II yields two scores, the Mental Developmental Index (MDI) and the Psychomotor Developmental Index (PDI). Both scores are standardized with a Mean =100 and an SD=15. Testing was conducted by specially trained nurses at the Child Development Centre, Mahé. All study forms were shipped to the University of Rochester, where data were double-entered. Test reliabilities for the BSID-II were determined as previously described (Strain et al. 2008).

2.4 Genetic analyses

In this study, we selected genes encoding proteins with an important role in the GSH pathway for metabolising toxicants, including MeHg: two genes (*GCLC* and *GCLM*) encoding the rate-limiting enzyme for the synthesis of GSH (Lu 2013) as well as glutathione S-transferase

(*GSTP1*) respectively. The latter enzyme has been suggested to conjugate MeHg to GSH (Custodio et al. 2014). The selected SNPs included rs761142 (*GCLC*), rs41303970 (*GCLM*) and rs1695 (*GSTP1*) and are presented in Table 1. SNPs were selected based on a careful review of published literature (Llop et al. 2015) and we included only SNPs that had been shown to influence expression/regulation of the corresponding gene (i.e. rs761142 in *GCLC* and rs41303970 in *GCLM*) and/or main effect associations with Hg biomarker concentrations (i.e. rs41303970 in *GCLM* and rs1695 in *GSTP1*). In addition SNPs were selected with consideration to previously reported minor allele frequencies (MAFs) of relevant populations (i.e. African, Asian and European populations) and only SNPs with MAFs >5% were included in the study. *GSTP1* rs1138272 was also considered but not included in the analyses due to low allele frequency (<1%) from preliminary genotyping of the NC1 cohort. This is in line with publicly available allele frequencies (http://www.ensembl.org) of this SNP in African populations (1%).

DNA was extracted from maternal blood using the Qiagen DNA Blood Mini kit (Qiagen, Hilden, Germany). *GCLC* rs761142 and *GSTP1* rs1695 were genotyped by TaqMan real-time PCR using custom assays from Thermo Scientific (Assay IDs C__2959418_20 and C_3237198_20 respectively). Reactions were analyzed on the ABI 7900HT Fast Real Time PCR System (Applied Biosystems, Thermo Fisher, Waltham, USA), using manufacturer's recommended standard conditions.

Due to the presence of several polymorphisms in the near vicinity of *GCLM* rs41303970, which prevented the design of optimal TaqMan assays, this SNP was instead genotyped by pyrosequencing. The assay was designed by PyroMark Assay Design 2.0 software (Qiagen) and included the following primer sequences: forward 5'CTGGCGGTCAGAGGACAG (biotinylated), reverse 5'GTGTAGGAAGCCCACCCTG and sequencing primer 5'TGGGCGGAGCCGCGA. Primers target sequences flank the repeat, which allows the generation of a specific PCR product for sequencing. PCR was performed using PyroMark PCR reagents (Qiagen) according to manufacturer's instructions and with negative controls included in each round of PCR. The PCR product was purified using Streptavidin Sepharose High Performance beads (Amersham Biosciences, UK) and pyrosequencing was carried out using the PyroMark reagents and PSQ HS96 Pyrosequencing System (Qiagen) according to manufacturer's protocol.

For quality control of genotyping data, >5% of samples were re-analyzed for all SNPs in a separate round of experiments with a 100% agreement between duplicates. Data quality was

also assessed by evaluating Hardy-Weinberg equilibrium using the conventional Chi-Square test.

2.5 Statistical analyses

Regression and analysis of variance (ANOVA) models for associations between SNPs and outcomes were performed based on an *a priori* analysis plan and all associations were evaluated using two-sided tests of significance at the $\alpha = 0.05$ level. The associations for the combination of the *GCLC* and *GCLM* polymorphisms were also evaluated, since both of these genes are required to constitute a functional GCL protein.

Under the assumption that fish consumption patterns and other determinants of MeHg exposure are similar for mothers with different SNPs, we used one-way ANOVA to estimate the association between each of the SNPs and Hg concentrations in the three biomarkers, in separate models. We used a 2 degree of freedom test to evaluate differences in hair, maternal blood, and cord Hg across the three levels of each SNP. We have seen that self-reported fish consumption is not well correlated to biomarkers of Hg and long chain PUFA in our cohorts. In this sample, the correlations between estimated fish consumption during pregnancy and maternal blood Hg (Spearman correlation coefficient=0.110), cord blood Hg (0.087), and prenatal hair Hg (0.047) are also small. Therefore, fish consumption cannot confound the relationship between the biomarkers of mercury and the genotypes and were not included in the analysis. Multiple linear regression was used to estimate the association of SNPs with BSID-II scores, adjusting for covariates previously chosen to cover the most important determinants of neurocognitive development in children (Strain et al. 2015). The covariates were child sex, maternal age at delivery, presence of two parents in the household, Hollingshead socioeconomic score, and child age at testing. These models for the BSID-II MDI and PDI, considered primary models, did not adjust for Hg because it is a potential mediator that would affect our ability to estimate the direct association between SNPs and BSID-II scores. Because no adjustment for Hg was made in these analyses, missing values for maternal hair Hg do not impact the number of observations included in the models. Therefore, our sample sizes for analyses between SNPs and BSID-II scores, which did not consider Hg variables, were considerably larger than those reported by Strain et al. (2015), in which Hg was the primary variable of interest. Since cord Hg values were missing for many subjects, we also repeated the maternal blood Hg analyses on the subsample of children with cord Hg samples.

To investigate whether polymorphisms in the GSH pathway could influence the relationship between maternal blood and cord blood Hg biomarker concentrations and neurodevelopment, we analyzed the interaction between SNPs and Hg biomarker concentrations on neurodevelopmental outcomes. In these secondary models for the BSID-II MDI and PDI, each biomarker for Hg was included as a covariate and we fit models with and without interactions of Hg and SNPs. Statistical analyses were undertaken using R (version 3.3.2; The R Foundation for Statistical Computing).

3 Results

3.1 Genetic characteristics

All SNPs analyzed were in Hardy Weinberg equilibrium. SNP information and minor allele frequencies (MAFs) of NC2 in comparison with related populations are presented in Table 1. *GCLM* and *GCLC* MAFs were similar to other African populations however, *GSTP1* for the Seychellois mothers showed a somewhat lower frequency (40% vs. 48%).

3.2 Correlation between Hg biomarker concentrations and associations with neurodevelopmental outcomes

Study population characteristics for the BSID-II models are presented in Table 2. Maternal hair Hg concentrations have previously been presented for this cohort and showed no association with child neurodevelopment (Strain et al., 2015), but the maternal blood and cord blood data have not been presented elsewhere. The correlations of maternal hair to maternal blood and cord blood Hg were 0.453 and 0.372 respectively, and the correlation between maternal and cord blood Hg was 0.664. No significant associations were observed between maternal blood or cord blood Hg with MDI scores (β=-0.0010, p=0.97 and β=0.0005, p=0.98 respectively) or PDI scores (β=-0.0041, p=0.88 and β=0.0289, p=0.092 respectively).

3.3 Associations of glutathione-related SNPs with maternal Hg concentrations

Based on the functional effect of variant alleles, *i.e.* either lower gene expression or lower enzyme activity (Table 1), we hypothesized that carriers of the minor alleles would show higher Hg concentrations, *i.e.* a less efficient MeHg metabolism. However, in contrast to this expectation, there was a significant negative association (lower hair Hg) for the *GCLC* rs761142 rare allele G with maternal hair Hg (Table 3, Figure 1). Mothers homozygous for the rare allele (genotype GG) had 0.61 ppm lower adjusted mean maternal hair Hg on average

compared to those who were homozygous for the common allele (genotype TT). We also observed a non-significant (p=0.17) negative association (lower hair Hg) between the *GCLM* rs41303970 rare allele (T) and maternal hair Hg, with homozygous (genotype TT) having 0.56 ppm lower adjusted mean hair Hg on average compared to CC. Combining the rs761142 and rs41303970 genotypes increased the strength of associations between GCL genotype and maternal hair Hg; carriers of a rare allele in both genotypes (*GCLC*:*GCLM* combination GG/TG:TT/TC) showed on average a 0.87 ppm decrease in maternal hair Hg concentrations compared to individuals homozygous for both common alleles (TT:CC) ($p<0.001$, Table 3, Figure 1). There were no associations between *GSTP1* rs1695 and maternal hair Hg and we did not observe any associations between the three SNPs and maternal blood Hg or cord blood Hg concentrations (Table 3). Associations between maternal hair Hg and *GCLC*, and the combination *GCLC* and *GCLM* remained significant in models fit to the smaller subset of subjects for which cord blood Hg values were available.

3.4 Associations of GSTP1 rs1695 with early cognitive and psychomotor development

Next we evaluated the influence of polymorphisms in the GSH pathway with early mental and motor development in children. The *GSTP1* rs1695 rare allele G showed a significantly negative association with MDI scores (p=0.048) and a non-significant negative association with PDI scores (p=0.089). The rare allele homozygotes (GG) scored on average 1.5 points lower on the MDI scores and 1.7 points lower on the PDI scores compared to common allele homozygotes (genotype AA) (Table 4, Fig 2). We did not observe any primary associations for *GCLM* rs41303970 or *GCLC* rs761142 with mental or psychomotor development in the children.

3.5 Association between Hg biomarker concentrations and neurodevelopment is modified by SNPs in GCLC and GSTP1

We observed significant interactions of *GCLC* rs761142 with maternal blood Hg (p=0.002) and cord blood Hg (p=0.014) in the covariate-adjusted association with PDI scores (secondary associations; Figure 3A and B, Supplemental Table 1). For children of mothers with the TT genotype (associated with high Hg concentrations in maternal hair), there was a negative association with PDI scores for maternal blood Hg (β=-0.07 [CI -0.15, 0.01]) and cord blood Hg (β=-0.07 [CI -0.12, -0.03]), while for children of mothers with the GG genotype (associated with low Hg concentrations in maternal hair) the associations with the PDI scores

were positive (β=0.23 [CI 0.08, 0.39] for maternal blood Hg and β=0.08 [CI -0.02, 0.18] for cord blood).

There were also significant interactions between *GSTP1* rs1695 genotype and maternal hair Hg on the MDI scores (p=0.03). There was a stronger negative association between maternal hair Hg and the MDI scores for children of mothers with the GG genotype (β=-0.57 [CI -1.02, -0.12]) compared to AA (β=-0.15 [CI -0.44, 0.15]; Figure 3C, Supplemental Table 1). None of the other secondary models considered had a significant interaction.

4 Discussion

In this study of a population eating a fish-rich diet, we have shown that maternal genotype of GSH-related genes is associated with both Hg concentrations in the mother's hair and early neurodevelopmental outcomes in the child

In contrast to our expectations, we observed a negative association of the *GCLC* rs761142 rare allele G with maternal hair Hg and the association increased in strength in combination with the *GCLM* rs41303970 rare allele T. However, there were no association of these SNPs with maternal or cord blood Hg whereas associations of SNPs with hair Hg remained significant among the subset of subjects with cord blood measures. The fewer associations of SNPs with blood Hg compared to hair Hg suggests that blood Hg is less influenced by genetic factors than hair Hg. We have previously shown, in this same cohort, that genetic variation in ABC transporter genes also show associations with maternal hair Hg (Engstrom et al. 2016) implying that genetics needs to be taken into consideration when using hair Hg as a biomarker of MeHg exposure. This finding is reflects what is seen for other metal biomarkers that in some cases show a significant influence of genetics, e.g. for manganese concentrations in blood (Wahlberg et al. 2016) and teeth (Wahlberg et al. 2017), as well as for urinary arsenic metabolites used as proxy for inorganic arsenic exposure (Schlawicke Engstrom et al. 2007). Although we did not find any associations between the SNPs and either maternal or cord blood Hg, this does not rule out a possible genetic influence of GSH pathway polymorphisms on these biomarkers. Previous studies of *GCLM* rs41303970 with Hg body-burden showed positive associations (higher Hg) of the rare allele with Hg in erythrocytes (Schlawicke Engstrom et al. 2008) and with Hg in blood (Barcelos et al. 2013; Harari et al. 2012; Schlawicke Engstrom et al. 2008), whereas, associations with faster Hg elimination (Harari et al. 2012) and lower Hg concentrations in blood have also been found (de Oliveira et al. 2014).

Another factor that could have masked potential association of GSH SNPs with maternal blood Hg concentrations is the expansion of the blood compartment during pregnancy which may cause larger variations in plasma volumes between individuals and thus influence blood Hg concentrations.

GCLC rs761142 is an intronic SNP that has been associated with reduced *GCLC* mRNA in human livers and lymphocytes (Wang et al. 2012). SNP rs41303970, which is situated in the *GCLM* promoter region, and has been associated with reduced transcriptional promoter activity (Nakamura et al. 2002). Thus, the functional consequences predicted from these two SNPs would be lower GCLM and GCLC protein levels for the variant allele carriers which we hypothesized could lead to reduced GSH synthesis, impaired Hg elimination and ultimately more Hg retained in the body. In contrast we found that variant allele carriers of *GCLM* and *GCLC* SNPs correlated with lower Hg levels in hair and no associations with blood Hg levels. Conflicting results for polymorphisms in *GCLC* and *GCLM*, have been reported among several studies (Llop et al, 2015) which, as suggested by Wang et al (2012), could indicate tissue/cell or environmental specificity of regulatory SNPs in *GCL*C and *GCLM*. Evidence for tissue-specific regulation of the *GCLC* rs761142, where the variant allele shows higher expression in some tissues and lower expression in others, can be found in the GTEx Portal data base (data were obtained from the GTEx Portal www.gtex.org on 02/15/18). For *GCLM* rs41303970, all tissues show lower expression for variant carriers. Samples suitable for gene-expression analyses will be necessary to investigate this hypothesis further, but were not available for the present study. Our findings also highlight the need for additional functional studies to differentiate unique aspects of MeHg transport and fate in hair versus blood.

In addition to the associations of *GCLC* rs761142 with hair Hg levels alone, the associations between Hg in maternal and cord blood and early motor development in children were significantly different in *GCLC* rs761142 carriers. While a weak negative association between Hg concentrations and PDI was observed for the common allele homozygotes, the rare allele homozygotes showed instead a positive association, indicating that this SNP may be protecting against Hg exposure in the infant. This unanticipated positive association of Hg and neurodevelopmental outcome suggests that additional factors that influence MeHg distribution within body compartments or toxicodynamics at the target organ (e.g. the brain) can overcome the toxicity implicated by a measure of body burden inferred from a given biomarker. Another explanation could be that the interaction is instead a reflection of the

child's own genotype that influences Hg elimination after birth and subsequent neurodevelopment in the infant.

For *GSTP1* rs1695 we did not observe an association between genotype and any of the Hg biomarker concentrations. Instead we observed a weak negative influence of the rare allele on neurodevelopment as well as negative interaction of the rare allele with maternal hair Hg on mental development, which implies that this allele may increase the sensitivity to Hg exposure in the child. The *GSTP1* rs1695 rare allele (G) causes a substitution of isoleucine (Ile) with valine (Val), which has shown to cause lower catalytic activity of the enzyme (Ali-Osman et al. 1997; Vorojeikina et al. 2017). In some population studies, the rare allele has been associated with lower Hg in hair (Goodrich et al. 2011) and blood (Schlawicke Engstrom et al. 2008, Parajuli et al 2016); however, there are also several studies in which associations between this SNP and Hg retention have been assessed without showing any significant effects (Barcelos et al. 2013; Custodio et al. 2005; Engstrom et al. 2011). The absence of an association between *GSTP1* rs1695 and Hg biomarkers implies that the association of this SNP with neurological development may be mediated by mechanisms other than Hg kinetics. This hypothesis is supported by the findings from a recent study assessing developmental effects of MeHg in *Drosophila* expressing variants of human *GSTP1*. In flies, where wild type *GSTP1* expression induces MeHg tolerance, the protein encoded by the rs1695 rare allele proved less enzymatically active and required higher expression levels to achieve MeHg tolerance to the same level as the wild type GSTP1 (Vorojeikina et al. 2017). Interestingly, the protective effects of GSTP1 expression were not seen to strictly correlate with reduced Hg body burden in Drosophila, as Hg body burden was seen to vary depending on the target tissue in which GSTP1 was expressed (Vorojeikina et al. 2017). Consistent with our findings here, these results suggest GSTP1 is likely to influence Hg toxicodynamics rather than kinetics. In addition to its role in GSH conjugation, GSTP1 is also an important factor in the defense against oxidative stress (Sanchez-Gomez et al. 2016), and thus, the less active variant of enzyme may increase susceptibility to Hg induced oxidative stress. Still, due to the small effect sizes observed for associations between *GSTP1* genotypes and developmental outcomes in this study, these associations need to be interpreted cautiously.

One strength of this study is the large size and unique cohort attributes of Seychellois mother and child pairs, which, due to their diet rich in fish and consequently high Hg concentrations, are well suited for studies of Hg toxicity and susceptibility factors. The cohort includes a

comprehensive data set which enables comparisons of Hg concentrations in different tissues with children's neurological outcomes. Limitations of this study are the candidate-gene-study-design which, in comparison to a genome wide approach, may provide a less complete picture of the influence of genetic polymorphisms on Hg toxicity. Another limitation is the lack of samples for gene-expression analyses which would have provided a further level of understanding of the mechanisms behind the observed associations.

In conclusion, our results indicate that maternal genetic variation in GSH related genes potentially influence maternal MeHg metabolism and may also modify associations between MeHg exposure and developmental outcomes. The findings contribute to increased understanding of the health impact of a fish-rich diet during pregnancy, and how this may differ not only between populations, but among individuals within a population. A next step for future studies is to examine the influence of children's genetic variation in GSH related genes on Hg toxicokinetics and dynamics during development.

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6 References

Ali-Osman F., Akande O., Antoun G., et al. 1997. Molecular cloning, characterization, and expression in *Escherichia coli* of full-length cDNAs of three human glutathione S-transferase Pi gene variants. Evidence for differential catalytic activity of the encoded proteins. J. Biol. Chem. 272, 10004-10012.

Andreoli V., Sprovieri F. 2017. Genetic aspects of susceptibility to mercury toxicity: An overview. Int. J. Environ. Res. Public Health 14(1) pii: E93.

412 Ballatori N., Clarkson T.W. 1985. Biliary secretion of glutathione and of glutathione-metal
413 complexes. Fundam. Appl. Toxicol. 5, 816-831.

414 Barcelos G.R., Grotto D., de Marco K.C., et al. 2013. Polymorphisms in glutathione-related
415 genes modify mercury concentrations and antioxidant status in subjects environmentally
416 exposed to methylmercury. Science Total Environ. 463-464, 319-325.

417 Cernichiari E., Toribara T.Y., Liang L., et al. 1995. The biological monitoring of mercury in
418 the Seychelles study. Neurotoxicology 16, 613-628.

419 Clarkson T.W., Magos L., Myers G.J. 2003. The toxicology of mercury - current exposures
420 and clinical manifestations. N. Engl. J. Med. 349, 1731-1737.

421 Costa L.G., Aschner M., Vitalone A., et al. 2004. Developmental neuropathology of
422 environmental agents. Ann. Rev. Pharmacol. Toxicol. 44, 87-110.

423 Custodio H.M., Broberg K., Wennberg M., al. 2004. Polymorphisms in glutathione-related
424 genes affect methylmercury retention. Arch. Environ. Occup. Health 59, 588-595.

425 Custodio H.M., Harari R., Gerhardsson L., et al. 2005. Genetic influences on the retention of
426 inorganic mercury. Arch. Environ. Occup. Health 60, 17-23.

427 Daniels J.L., Longnecker M.P., Rowland A.S., et al. 2004. Fish intake during pregnancy and
428 early cognitive development of offspring. Epidemiology 15, 394-402.

429 Davidson P.W., Myers G.J., Cox C., et al. 1998. Effects of prenatal and postnatal
430 methylmercury exposure from fish consumption on neurodevelopment: Outcomes at 66
431 months of age in the Seychelles Child Development Study. JAMA 280, 701-707.

432 de Oliveira A.A., de Souza M.F., Lengert A., et al. 2014. Genetic polymorphisms in
433 glutathione (GSH-) related genes affect the plasmatic Hg/whole blood Hg partitioning and the
434 distribution between inorganic and methylmercury levels in plasma collected from a fish-
435 eating population. BioMed Res. Int. 2014, 940952.

436 Engstrom K., Love T.M., Watson G.E., et al. 2016. Polymorphisms in ATP-binding cassette
437 transporters associated with maternal methylmercury disposition and infant
438 neurodevelopment in mother-infant pairs in the Seychelles Child Development Study.
439 Environ. Int. 94, 224-229.

440 Engstrom K.S., Wennberg M., Stromberg U., et al. 2011. Evaluation of the impact of genetic
441 polymorphisms in glutathione-related genes on the association between methylmercury or n-3

442 polyunsaturated long chain fatty acids and risk of myocardial infarction: A case-control study.
443 Environ. Health 10, 33.

444 Goodrich J.M., Wang Y., Gillespie B., et al. 2011. Glutathione enzyme and selenoprotein
445 polymorphisms associate with mercury biomarker levels in Michigan dental professionals.
446 Toxicol. Appl. Pharm. 257, 301-308.

447 Goodrich J.M., Basu N. 2012. Variants of glutathione s-transferase pi 1 exhibit differential
448 enzymatic activity and inhibition by heavy metals. Toxicol. In Vitro 26, 630-635.

449 Grandjean P., Weihe P., White R.F., et al. 1997. Cognitive deficit in 7-year-old children with
450 prenatal exposure to methylmercury. Neurotoxicol. Teratol. 19, 417-428.

451 Harari R., Harari F., Gerhardsson L., et al. 2012. Exposure and toxic effects of elemental
452 mercury in gold-mining activities in Ecuador. Toxicol. Lett. 213, 75-82.

453 Johansson C., Castoldi A.F., Onishchenko N., et al. 2007. Neurobehavioural and molecular
454 changes induced by methylmercury exposure during development. Neurotox. Res. 11, 241-
455 260.

456 Kaur P., Aschner M., Syversen T. 2006. Glutathione modulation influences methylmercury
457 induced neurotoxicity in primary cell cultures of neurons and astrocytes. Neurotoxicology 27,
458 492–500.

459 Llop S., Guxens M., Murcia M., et al. 2012. Prenatal exposure to mercury and infant
460 neurodevelopment in a multicenter cohort in Spain: Study of potential modifiers. Am. J.
461 Epidemiol. 175, 451-465.

462 Llop S., Ballester F., Broberg K. 2015. Effect of gene-mercury interactions on mercury
463 toxicokinetics and neurotoxicity. Curr. Env. Health Rep. 2, 179-194.

464 Lu S.C. 2013. Glutathione synthesis. Biochim. Biophys. Acta 1830, 3143-3153.

465 Nakamura S., Kugiyama K., Sugiyama S., et al. 2002. Polymorphism in the 5'-flanking region
466 of human glutamate-cysteine ligase modifier subunit gene is associated with myocardial
467 infarction. Circulation 105, 2968-2973.

468 Parajuli R.P., Goodrich J.M., Chou H.N., et al. 2016. Genetic polymorphisms are associated
469 with hair, blood, and urine mercury levels in the American Dental Association (ADA) study
470 participants. Environ Res. 149, 247-258.

Sanchez-Gomez F.J., Diez-Dacal B., Garcia-Martin E., et al. 2016. Detoxifying enzymes at the cross-roads of inflammation, oxidative stress, and drug hypersensitivity: Role of glutathione transferase P1-1 and aldose reductase. Front. Pharm. 7, 237.

Schlawicke Engstrom K., Broberg K., Concha G., et al. 2007. Genetic polymorphisms influencing arsenic metabolism: Evidence from Argentina. Environ. Health Perspect. 115, 599-605.

Schlawicke Engstrom K., Stromberg U., Lundh T., et al. 2008. Genetic variation in glutathione-related genes and body burden of methylmercury. Environ. Health Perspect. 116, 734-739.

Strain J.J., Davidson P.W., Bonham M.P., et al. 2008. Associations of maternal long-chain polyunsaturated fatty acids, methyl mercury, and infant development in the Seychelles Child Development Nutrition Study. Neurotoxicology 29, 776-782.

Strain J.J., Davidson P.W., Thurston S.W., et al. 2012. Maternal PUFA status but not prenatal methylmercury exposure is associated with children's language functions at age five years in the Seychelles. J. Nutr. 142, 1943-9.

Strain J.J., Yeates A.J., van Wijngaarden E., et al. 2015. Prenatal exposure to methyl mercury from fish consumption and polyunsaturated fatty acids: Associations with child development at 20 mo of age in an observational study in the Republic of Seychelles. Am. J. Clin. Nutr. 101, 530-537.

van Wijngaarden E., Thurston S.W., Myers G.J., et al. 2017. Methyl mercury exposure and neurodevelopmental outcomes in the Seychelles Child Development Study main cohort at age 22 and 24 years. Neurotoxicol. Teratol. 59, 35-42.

Vejrup K., Schjolberg S., Knutsen H.K., et al. 2016. Prenatal methylmercury exposure and language delay at three years of age in the Norwegian mother and child cohort study. Environ. Int. 92-93, 63-69.

Vorojeikina D., Broberg K., Love T.M., et al. 2017. Glutathione S-transferase activity moderates methylmercury toxicity during development in Drosophila. Toxicol. Sci. 157, 211-221.

Wahlberg K., Kippler M., Alhamdow A., et al. 2016. Common polymorphisms in the solute carrier SLC30A10 are associated with blood manganese and neurological function. Toxicol. Sci. 149, 473-483.

Wahlberg K., Arora M., Curtin A., et al. 2017. Polymorphisms in manganese transporters show developmental stage and sex specific associations with manganese concentrations in primary teeth. Neurotoxicology, [epub ahead of print] doi: 10.1016/j.neuro.2017.09.003.

Wang D., Curtis A., Papp A.C., et al. 2012. Polymorphism in glutamate cysteine ligase catalytic subunit (GCLC) is associated with sulfamethoxazole-induced hypersensitivity in HIV/aids patients. BMC Med. Genom. 5, 32.

Table 1. SNP information and minor allele frequencies (MAFs) in the study cohort of Seychellois mothers (NC2) compared to other populations

				MAF (%)			
Gene / Chromosome	**SNP / Alleles[a]**	**SNP type[b]**	**Functional effect of minor allele/Hypothesized effect on Hg concentrations**	**NC2**	**Africa[c]**	**South Asia[c]**	**Europe[c]**
GCLM 1	rs41303970 C/T	Upstream variant	Reduced *GCLM* promoter activity (Nakamura et al. 2002)/Higher Hg	23	22	9	15
GCLC 6	rs761142 T/G	Intronic variant	Reduced *GCLC* gene-transcription (Wang et al. 2012)/Higher Hg	34	37	37	27
GSTP1 11	rs1695 A/G	Missense Ile/Val	Reduced enzyme activity (Ali-Osman et al. 1997; Goodrich and Basu 2012)/Higher Hg	40	48	30	33
GSTP1 11	rs1138272 C/T	Missense Ala/Val	Reduced enzyme activity (Ali-Osman et al. 1997; Goodrich and Basu 2012)/Higher Hg	N/A[d]	1	7	7

[a] Minor allele is denoted last.
[b] Source: the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov)
[c] Average MAFs for African, South Asian and European populations. Source: Ensembl Genome Browser (http://www.ensembl.org)
[d] *GSTP1* rs1138272 was considered but not included in the analyses due to low allele frequency (<1%) from preliminary genotyping of another cohort from Seychelles.

Table 2. Characteristics of study population including summary statistics of outcomes and covariates for mother/child pairs used in the BSID models. These data include 48% female children and 73% children living with two parents at the time of BSID testing.

Variable	N available	N missing	Mean	SD	Min	25 percentile	50 percentile	75 percentile	Max
Maternal hair Hg (ppm)	1230	100	3.87	3.43	0	1.44	2.88	5.13	31.66
Maternal blood Hg (μg/L)	1266	64	18.22	10.86	1.87	10.84	15.80	22.83	84.15
Cord blood Hg (μg/L)	935	395	34.48	20.46	1.91	20.01	30.15	43.91	181.27
MDI at 20 months	1326	4	87.6	10.7	49	82	88	94	118
PDI at 20 months	1324	6	96.7	10.6	49	90	97	104	136
SES	1330	0	32.0	10.35	11	24	31.5	39.5	63
Maternal age at delivery (years)	1330	0	27.1	6.3	16.3	22.1	26.1	31.5	44.8
Child test age (months)	1330	0	20.3	1.4	15	20	20	21	32

Table 3. Associations of genotypes with MeHg biomarker concentrations. When the mean MeHg concentrations differ significantly across genotype levels based on a 2 df test (p-value 2), the mean differences from the reference genotype (β) are also given with their p-values (p 1).

		Maternal Hair					**Maternal Blood**			**Cord blood**		
Genes	**Genotypes**	**Mean**	**CI**	β	**p 1**	**n**	**Mean**	**CI**	**n**	**Mean**	**CI**	**n**
GCLM	CC	4.07	(3.82,4.32)			722	18.28	(17.50,19.06)	808	34.84	(33.13,36.55)	557
	CT	3.63	(3.31,3.96)			427	18.46	(17.44,19.48)	486	34.34	(32.11,36.57)	326
	TT	3.22	(2.40,4.05)			67	16.74	(14.11,19.38)	72	31.64	(25.50,37.78)	43
p-value 2		0.166					0.649			0.529		
GCLC	TT	4.12	(3.83,4.42)			527	18.43	(17.51,19.35)	585	34.44	(32.43,36.45)	395
	TG	3.73	(3.44,4.01)	-0.46	**0.02**	562	18.01	(17.14,18.89)	637	34.59	(32.67,36.52)	430
	GG	3.52	(2.95,4.09)	-0.61	0.06	138	18.28	(16.47,20.08)	155	33.60	(29.74,37.45)	107
p-value 2		**0.037**					0.776			0.876		
GCLC/M	TT&CC	4.40	(4.01,4.79)			296	18.40	(17.17,19.63)	327	35.41	(32.71,38.11)	219
	G-&CC	3.86	(3.54,4.19)	-0.58	**0.02**	423	18.18	(17.17,19.20)	478	34.29	(32.11,36.47)	335
	TT&T-	3.74	(3.29,4.19)	-0.52	0.08	225	18.46	(17.04,19.88)	253	33.20	(30.15,36.24)	172
	G-&T-	3.44	(3.03,3.85)	-0.87	**0.00**	269	18.05	(16.76,19.34)	305	34.75	(31.90,37.59)	197
p-value 2		**0.018**					0.839			0.766		
GSTP1	AA	3.82	(3.50,4.14)			446	18.44	(17.44,19.44)	502	33.75	(31.54,35.95)	333
	AG	3.92	(3.64,4.20)			580	18.29	(17.41,19.16)	653	35.10	(33.20,36.99)	450
	GG	3.82	(3.34,4.29)			202	17.64	(16.17,19.11)	223	34.27	(31.00,37.54)	151
p-value 2		0.968					0.643			0.766		

Table 4. Associations of genotypes with developmental outcomes adjusted for covariates.[a] When the outcome means differ significantly across genotype levels based on a 2 df test (p-value 2), the mean differences from the reference genotype (β) are also given with their p-values (p 1).

		MDI					**PDI**		
Genes	**Genotypes**	**Mean**	**CI**	**β**	**p 1**	**n**	**Mean**	**CI**	**n**
GCLM	CC	87.75	(87.02,88.48)			783	96.68	(95.94,97.42)	782
	CT	87.41	(86.46,88.37)			458	96.75	(95.78,97.72)	457
	TT	86.47	(84.03,88.92)			70	96.44	(93.96,98.93)	70
p-value 2		0.690					0.995		
GCLC	TT	87.65	(86.79,88.51)			564	96.98	(96.11,97.85)	562
	TG	87.58	(86.75,88.41)			610	96.63	(95.80,97.47)	611
	GG	87.42	(85.74,89.09)			149	95.75	(94.05,97.45)	148
p-value 2		0.759					0.371		
GCLC/M	TT&CC	88.07	(86.92,89.22)			317	96.60	(95.43,97.77)	315
	G-&CC	87.49	(86.54,88.44)			463	96.66	(95.70,97.63)	464
	TT&T-	87.07	(85.75,88.39)			241	97.39	(96.05,98.73)	241
	G-&T-	87.49	(86.28,88.70)			287	96.13	(94.91,97.36)	286
p-value 2		0.721					0.353		
GSTP1	AA	88.60	(87.67,89.54)			477	97.50	(96.56,98.45)	475
	AG	87.02	(86.21,87.84)	-1.59	**0.012**	626	96.36	(95.54,97.18)	626
	GG	87.12	(85.75,88.50)	-1.48	0.079	221	95.83	(94.44,97.22)	221
p-value 2		**0.048**					0.089		

[a] The models were adjusted for child sex, maternal age at delivery, presence of two parents in the household, Hollingshead socioeconomic score, and child age at testing.

Figure legends

Figure 1. Associations of *GCLM* rs41303970 (A) and *GCLC* rs761142 (B) genotypes separately and in the combination rs761142:rs41303970 (C) with maternal prenatal Hg hair concentrations including 95% confidence intervals (CI). To simplify combinations of *GCLC* and *GCLM* genotypes, heterozygotes and rare allele homozygotes for each SNP were combined into groups representing rare allele carriers. *p≤0.10, **p≤0.05, ***p≤0.01

Figure 2. Associations between *GSTP1* rs1695 genotypes and children's mental development index (A) and motor development index (B) at 20 months. *p≤0.10, **p≤0.05, ***p≤0.01

Figure 3. Associations between Hg biomarker concentrations and children's neurological development showing significant differences in slopes across genotype levels. (A) Association between maternal blood Hg and the PDI with separate slopes by levels of *GCLC* rs761142, (B) association between maternal blood Hg and the PDI with separate slopes by levels of *GCLC* rs761142, and (C) association between maternal hair Hg and the MDI with separate slopes by levels of *GSTP1* rs1695.