



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

4

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29 **Abstract**

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

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

41 **Results:** *GCLC* rs761142 TT homozygotes showed statistically higher mean maternal hair Hg  
42 (4.12 ppm) than G carriers (AG 3.73 and GG 3.52 ppm) ( $p=0.037$ ). For the combination of  
43 *GCLC* rs761142 and *GCLM* rs41303970, double homozygotes TT+CC showed higher hair  
44 Hg (4.40 ppm) than G+T carriers (3.44 ppm;  $p=0.018$ ). No associations were observed  
45 between *GSTP1* rs1695 and maternal hair Hg or between any genotypes and maternal blood  
46 Hg or cord blood Hg. The maternal *GSTP1* rs1695 rare allele (G) was associated with a lower  
47 MDI among children ( $\beta=-1.48$ ,  $p=0.048$ ). We also observed some interactions: increasing Hg  
48 in maternal and cord blood was associated with lower PDI among *GCLC* rs761142 TT  
49 carriers; and increasing Hg in hair was associated with lower MDI among *GSTP1* rs1695 GG  
50 carriers.

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

55

56 **Keywords:** Methylmercury, GCLC, GCLM, GSTP1, neurodevelopment

57

## 58 **1 Introduction**

59 Fish is the main source of human low-level methylmercury (MeHg) exposure. At high levels,  
60 MeHg has clear detrimental effects on the nervous system (Clarkson et al. 2003), but the  
61 neurotoxic effects of low-level exposure are not established. The developing brain is  
62 particularly sensitive to neurotoxicants including MeHg (Costa et al. 2004; Johansson et al.  
63 2007), but it is unclear at what MeHg level the fetal brain is affected. Consequently, it is  
64 unclear if fish ingestion poses a risk for fetal toxicity. Research results of MeHg exposure  
65 from fish consumption in relation to neurodevelopmental outcomes in children have been  
66 contradictory between studies of different populations, with adverse associations observed in  
67 some studies (Grandjean et al. 1997; Vejrup et al. 2016), but not in others (Daniels et al.  
68 2004; Davidson et al. 1998; Llop et al. 2012; Strain et al. 2015). Several studies have  
69 suggested that genetics may contribute to MeHg body burden as well as to defense  
70 mechanisms against MeHg toxicity (Andreoli and Sprovieri 2017; Llop et al. 2015).

71 An important mechanism in MeHg metabolism involves the conjugation of MeHg to the  
72 small tripeptide glutathione (GSH), which facilitates elimination of the conjugate in the bile  
73 via the ABC-transporter system (Ballatori and Clarkson 1985). The rate-limiting enzyme for  
74 GSH synthesis is  $\gamma$ -glutamyl-cysteine ligase (GCL), which is composed of a catalytic subunit  
75 (GCLC) and a modifier subunit (GCLM) (Lu 2013). Further, the conjugation of GSH to  
76 MeHg has been suggested to be catalyzed by glutathione S-transferases, particularly the pi 1  
77 isoform (GSTP1) (Custodio et al. 2004). Genetic polymorphisms in *GCLC*, *GCLM*, and  
78 *GSTP1* have been linked to MeHg retention and body burden in adults (Barcelos et al. 2013;  
79 Custodio et al. 2004; Goodrich et al. 2011; Parajuli et al. 2016; Schlawicke Engstrom et al.  
80 2008). In addition, our group has recently shown that GSTP1 polymorphisms, expressed in  
81 *Drosophila*, may influence MeHg toxicity during development through both toxicokinetic and  
82 toxicodynamic mechanisms (Vorojeikina et al. 2017).

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

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

95

## 96 **2 Materials and methods**

### 97 ***2.1 Study population***

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

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

123 Additionally, participants without genetic data and one each of thirteen sibling pairs were  
124 excluded. There were 1330 pairs eligible for models for the BSID endpoints, 1230, 1266, and  
125 935 of whom had samples of hair, maternal and cord blood respectively. The study was  
126 conducted according to guidelines laid down in the Declaration of Helsinki and all study  
127 procedures involving participants were reviewed and approved by the Seychelles Ethics  
128 Board, the Research Subjects Review Board at the University of Rochester, and the Regional  
129 Ethics Committee at Lund University, Sweden.

## 130 **2.2 Hg measurements**

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

## 143 **2.3 Neurodevelopmental assessment**

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

## 151 **2.4 Genetic analyses**

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

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

168 DNA was extracted from maternal blood using the Qiagen DNA Blood Mini kit (Qiagen,  
169 Hilden, Germany). *GCLC* rs761142 and *GSTP1* rs1695 were genotyped by TaqMan real-time  
170 PCR using custom assays from Thermo Scientific (Assay IDs C\_\_2959418\_20 and  
171 C\_3237198\_20 respectively). Reactions were analyzed on the ABI 7900HT Fast Real Time  
172 PCR System (Applied Biosystems, Thermo Fisher, Waltham, USA), using manufacturer's  
173 recommended standard conditions.

174 Due to the presence of several polymorphisms in the near vicinity of *GCLM* rs41303970,  
175 which prevented the design of optimal TaqMan assays, this SNP was instead genotyped by  
176 pyrosequencing. The assay was designed by PyroMark Assay Design 2.0 software (Qiagen)  
177 and included the following primer sequences: forward 5'CTGGCGGTTCAGAGGACAG  
178 (biotinylated), reverse 5'GTGTAGGAAGCCCACCCTG and sequencing primer  
179 5'TGGGCGGAGCCGCGA. Primers target sequences flank the repeat, which allows the  
180 generation of a specific PCR product for sequencing. PCR was performed using PyroMark  
181 PCR reagents (Qiagen) according to manufacturer's instructions and with negative controls  
182 included in each round of PCR. The PCR product was purified using Streptavidin Sepharose  
183 High Performance beads (Amersham Biosciences, UK) and pyrosequencing was carried out  
184 using the PyroMark reagents and PSQ HS96 Pyrosequencing System (Qiagen) according to  
185 manufacturer's protocol.

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

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

## 190 **2.5 Statistical analyses**

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

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



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

227

## 228 **3 Results**

### 229 ***3.1 Genetic characteristics***

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

### 234 ***3.2 Correlation between Hg biomarker concentrations and associations with*** 235 ***neurodevelopmental outcomes***

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

### 244 ***3.3 Associations of glutathione-related SNPs with maternal Hg concentrations***

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

251 compared to those who were homozygous for the common allele (genotype TT). We also  
252 observed a non-significant ( $p=0.17$ ) negative association (lower hair Hg) between the *GCLM*  
253 rs41303970 rare allele (T) and maternal hair Hg, with homozygous (genotype TT) having  
254 0.56 ppm lower adjusted mean hair Hg on average compared to CC. Combining the rs761142  
255 and rs41303970 genotypes increased the strength of associations between GCL genotype and  
256 maternal hair Hg; carriers of a rare allele in both genotypes (*GCLC*:*GCLM* combination  
257 GG/TG:TT/TC) showed on average a 0.87 ppm decrease in maternal hair Hg concentrations  
258 compared to individuals homozygous for both common alleles (TT:CC) ( $p<0.001$ , Table 3,  
259 Figure 1). There were no associations between *GSTP1* rs1695 and maternal hair Hg and we  
260 did not observe any associations between the three SNPs and maternal blood Hg or cord  
261 blood Hg concentrations (Table 3). Associations between maternal hair Hg and *GCLC*, and  
262 the combination *GCLC* and *GCLM* remained significant in models fit to the smaller subset of  
263 subjects for which cord blood Hg values were available.

#### 264 ***3.4 Associations of GSTP1 rs1695 with early cognitive and psychomotor development***

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

#### 273 ***3.5 Association between Hg biomarker concentrations and neurodevelopment is modified*** 274 ***by SNPs in GCLC and GSTP1***

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

282 were positive ( $\beta=0.23$  [CI 0.08, 0.39] for maternal blood Hg and  $\beta=0.08$  [CI -0.02, 0.18] for  
283 cord blood).

284 There were also significant interactions between *GSTP1* rs1695 genotype and maternal hair  
285 Hg on the MDI scores ( $p=0.03$ ). There was a stronger negative association between maternal  
286 hair Hg and the MDI scores for children of mothers with the GG genotype ( $\beta=-0.57$  [CI -1.02,  
287 -0.12]) compared to AA ( $\beta=-0.15$  [CI -0.44, 0.15]; Figure 3C, Supplemental Table 1). None of  
288 the other secondary models considered had a significant interaction.

289

#### 290 **4 Discussion**

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

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

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

318 *GCLC* rs761142 is an intronic SNP that has been associated with reduced *GCLC* mRNA in  
319 human livers and lymphocytes (Wang et al. 2012). SNP rs41303970, which is situated in the  
320 *GCLM* promoter region, and has been associated with reduced transcriptional promoter  
321 activity (Nakamura et al. 2002). Thus, the functional consequences predicted from these two  
322 SNPs would be lower *GCLM* and *GCLC* protein levels for the variant allele carriers which  
323 we hypothesized could lead to reduced GSH synthesis, impaired Hg elimination and  
324 ultimately more Hg retained in the body. In contrast we found that variant allele carriers of  
325 *GCLM* and *GCLC* SNPs correlated with lower Hg levels in hair and no associations with  
326 blood Hg levels. Conflicting results for polymorphisms in *GCLC* and *GCLM*, have been  
327 reported among several studies (Llop et al, 2015) which, as suggested by Wang et al (2012),  
328 could indicate tissue/cell or environmental specificity of regulatory SNPs in *GCLC* and  
329 *GCLM*. Evidence for tissue-specific regulation of the *GCLC* rs761142, where the variant  
330 allele shows higher expression in some tissues and lower expression in others, can be found in  
331 the GTEx Portal data base (data were obtained from the GTEx Portal [www.gtexp.org](http://www.gtexp.org) on  
332 02/15/18). For *GCLM* rs41303970, all tissues show lower expression for variant carriers.  
333 Samples suitable for gene-expression analyses will be necessary to investigate this hypothesis  
334 further, but were not available for the present study. Our findings also highlight the need for  
335 additional functional studies to differentiate unique aspects of MeHg transport and fate in hair  
336 versus blood.

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

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

349 For *GSTP1* rs1695 we did not observe an association between genotype and any of the Hg  
350 biomarker concentrations. Instead we observed a weak negative influence of the rare allele on  
351 neurodevelopment as well as negative interaction of the rare allele with maternal hair Hg on  
352 mental development, which implies that this allele may increase the sensitivity to Hg  
353 exposure in the child. The *GSTP1* rs1695 rare allele (G) causes a substitution of isoleucine  
354 (Ile) with valine (Val), which has shown to cause lower catalytic activity of the enzyme (Ali-  
355 Osman et al. 1997; Vorojeikina et al. 2017). In some population studies, the rare allele has  
356 been associated with lower Hg in hair (Goodrich et al. 2011) and blood (Schlawicke  
357 Engstrom et al. 2008, Parajuli et al 2016); however, there are also several studies in which  
358 associations between this SNP and Hg retention have been assessed without showing any  
359 significant effects (Barcelos et al. 2013; Custodio et al. 2005; Engstrom et al. 2011). The  
360 absence of an association between *GSTP1* rs1695 and Hg biomarkers implies that the  
361 association of this SNP with neurological development may be mediated by mechanisms  
362 other than Hg kinetics. This hypothesis is supported by the findings from a recent study  
363 assessing developmental effects of MeHg in *Drosophila* expressing variants of human  
364 *GSTP1*. In flies, where wild type *GSTP1* expression induces MeHg tolerance, the protein  
365 encoded by the rs1695 rare allele proved less enzymatically active and required higher  
366 expression levels to achieve MeHg tolerance to the same level as the wild type *GSTP1*  
367 (Vorojeikina et al. 2017). Interestingly, the protective effects of *GSTP1* expression were not  
368 seen to strictly correlate with reduced Hg body burden in *Drosophila*, as Hg body burden was  
369 seen to vary depending on the target tissue in which *GSTP1* was expressed (Vorojeikina et al.  
370 2017). Consistent with our findings here, these results suggest *GSTP1* is likely to influence  
371 Hg toxicodynamics rather than kinetics. In addition to its role in GSH conjugation, *GSTP1* is  
372 also an important factor in the defense against oxidative stress (Sanchez-Gomez et al. 2016),  
373 and thus, the less active variant of enzyme may increase susceptibility to Hg induced  
374 oxidative stress. Still, due to the small effect sizes observed for associations between *GSTP1*  
375 genotypes and developmental outcomes in this study, these associations need to be interpreted  
376 cautiously.

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

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

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

393

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404

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

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

<sup>a</sup> Minor allele is denoted last.

<sup>b</sup> Source: the National Centre for Biotechnology Information (<https://www.ncbi.nlm.nih.gov>)

<sup>c</sup> Average MAFs for African, South Asian and European populations. Source: Ensembl Genome Browser (<http://www.ensembl.org>)

<sup>d</sup> *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 ( $\mu\text{g/L}$ )	1266	64	18.22	10.86	1.87	10.84	15.80	22.83	84.15
Cord blood Hg ( $\mu\text{g/L}$ )	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 ( $\beta$ ) are also given with their p-values (p 1).

Genes	Genotypes	Maternal Hair					Maternal Blood			Cord blood		
		Mean	CI	$\beta$	p 1	n	Mean	CI	n	Mean	CI	n
<i>GCLM</i>	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		
<i>GCLC</i>	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	<b>0.02</b>	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		<b>0.037</b>					0.776			0.876		
<i>GCLC/M</i>	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	<b>0.02</b>	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	<b>0.00</b>	269	18.05	(16.76,19.34)	305	34.75	(31.90,37.59)	197
p-value 2		<b>0.018</b>					0.839			0.766		
<i>GSTP1</i>	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.<sup>a</sup> 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 ( $\beta$ ) are also given with their p-values (p 1).

Genes	Genotypes	MDI				PDI			
		Mean	CI	$\beta$	p 1	n	Mean	CI	n
<i>GCLM</i>	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		
<i>GCLC</i>	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		
<i>GCLC/M</i>	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		
<i>GSTP1</i>	AA	88.60	(87.67,89.54)			477	97.50	(96.56,98.45)	475
	AG	87.02	(86.21,87.84)	-1.59	<b>0.012</b>	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		<b>0.048</b>					0.089		

<sup>a</sup> 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.

1 **Figure legends**

2

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

8

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

11

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

18