



Modification of neurobehavioral effects of mercury by a genetic polymorphism of coproporphyrinogen oxidase in children

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ABSTRACT

Mercury (Hg) is neurotoxic, and children may be particularly susceptible to this effect. A current major challenge is the identification of children who may be uniquely susceptible to Hg toxicity because of genetic disposition. We examined the hypothesis that CPOX4, a genetic variant of the heme pathway enzyme coproporphyrinogen oxidase (CPOX) that affects susceptibility to mercury toxicity in adults, also modifies the neurotoxic effects of Hg in children. Five hundred seven children, 8–12 years of age at baseline, participated in a clinical trial to evaluate the neurobehavioral effects of Hg from dental amalgam tooth fillings in children. Subjects were evaluated at baseline and at 7 subsequent annual intervals for neurobehavioral performance and urinary mercury levels. Following the completion of the clinical trial, genotyping assays for CPOX4 allelic status were performed on biological samples provided by 330 of the trial participants. Regression modeling strategies were employed to evaluate associations between CPOX4 status, Hg exposure, and neurobehavioral test outcomes. Among girls, few significant CPOX4–Hg interactions or independent main effects for Hg or CPOX4 were observed. In contrast, among boys, numerous significant interaction effects between CPOX4 and Hg were observed spanning all 5 domains of neurobehavioral performance. All underlying dose–response associations between Hg exposure and test performance were restricted to boys with the CPOX4 variant, and all of these associations were in the expected direction where increased exposure to Hg decreased performance. These findings are the first to demonstrate genetic susceptibility to the adverse neurobehavioral effects of Hg exposure in children. The paucity of responses among same-age girls with comparable Hg exposure provides evidence of sexual dimorphism in genetic susceptibility to the adverse neurobehavioral effects of Hg in children and adolescents.

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1. Introduction

Children are recognized as having heightened susceptibility to the adverse effects of environmental chemicals, as compared with adults with similar exposures (Faustman et al., 2000; Landrigan and Goldman, 2011). Of particular concern in this respect are possible neurological deficits associated with mercury exposure (Clarkson, 2003; Echeverria et al., 1998; Goering et al., 1992), which may cause impairment of the developing central nervous system along with attendant

personality, cognitive function and behavioral disorders (Counter and Buchanan, 2004; Davidson et al., 2004; Levy et al., 2004). A current major challenge is the identification of those children who may be uniquely susceptible to Hg-mediated neurological deficits because of genetic predisposition.

Previous studies in adults have identified at least 4 commonly expressed genetic polymorphisms that modify the effects of Hg on a wide range of neurobehavioral functions (Echeverria et al., 2005, 2006, 2010; Heyer et al., 2004, 2008, 2009). Of particular interest in this respect is a single nucleotide polymorphism (A>C) (rs1131857) in exon 4 of the gene encoding an asparagine-to-histidine change at amino acid 272 (N272H) of the heme biosynthetic pathway enzyme, coproporphyrinogen oxidase (CPOX, EC 1.3.3.3). This variant, referred to herein as “CPOX4”, both increases sensitivity to the neurobehavioral effects of Hg (Echeverria et al., 2006) and modifies urinary porphyrin excretion as a potential biomarker of this effect (Woods et al., 2005; Li and Woods, 2009). The population frequencies of the homozygous

Abbreviations: Hg, mercury; IQ, intelligence quotient; CPOX, coproporphyrinogen oxidase.

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wildtype (A/A), heterozygous (A/C) and homozygous mutant (C/C) genotypes within this cohort were 0.72, 0.25, and 0.03, respectively, and were equally prevalent among males and females, suggesting substantial exposure to the CPOX4 variant.

In the present study, we examined the hypothesis that CPOX4 would modify the adverse neurobehavioral effects of Hg exposure in children as previously observed in adults. Subjects were children and adolescents who participated in a recently completed prospective randomized dental amalgam clinical trial between ages 8 and 18 and for whom longitudinal (annual) neurobehavioral assessments and quantitative measures of dental amalgam Hg exposure over 7 years of follow-up were available. Additionally, to preclude selection bias possibly associated with those genotyped for CPOX4 *per se*, we made comparable assessments with respect to second single nucleotide polymorphism located at exon 5 (rs1729995) (G>A) of the CPOX gene encoding a synonymous mutation in the CPOX enzyme (E330E), referred to herein as "CPOX5". CPOX5 has been previously identified as distributed among men and women within our adult dental population with frequencies of the homozygous common (wildtype), heterozygous, and homozygous mutant alleles of 0.48, 0.43 and 0.09, respectively (Woods et al., 2005). CPOX5 is not known to be in linkage disequilibrium with CPOX4. We made these assessments independently in boys and girls.

2. Methods

2.1. The study population

The current study was performed on a subset of 330 subjects who participated as children in the Casa Pia Dental Amalgam Clinical Trial (DeRouen et al., 2002, 2006) conducted between 1996 and 2006.

Participants in the clinical trial included 279 boys and 228 girls, aged 8–12 years at baseline, who were students of the Casa Pia school system in Lisbon, Portugal. Children were initially randomized to Hg amalgam (treatment) or composite resin (control) dental treatment groups. Subjects were evaluated at baseline and at 7 subsequent annual intervals following initial dental treatment using an extensive battery of neurobehavioral assessments (Slade et al., 2008; Townes et al., 2008a, 2008b). Follow-up data were obtained on a similar number of subjects in each treatment group. Studies conducted during the course of the clinical trial (Evens et al., 2001) demonstrated that the children had no significant exposure to methylmercury from dietary fish consumption. However, other unidentified sources of environmental mercury exposure may have contributed to baseline urinary mercury concentrations, which were 1.5 ± 1.2 (0.1–7.7) and 1.4 ± 1.1 (0.0–8.6) $\mu\text{g/L}$ for amalgam and composite groups, respectively. Mean urinary mercury concentrations by treatment group and by gender for each year of the clinical trial have been previously published (Woods et al., 2007).

2.2. Neurobehavioral tests employed

A comprehensive neurobehavioral test battery was used in this analysis, including measures from the Rays Verbal Learning Test (RAVALT), subtests from the Wide Range Assessment of Memory and Learning and Visual Motor Abilities (WRAVMA), the Wechsler Intelligence Scale for Children III (WISC-III), and the Wechsler Intelligence Scale for Adults-III (WMS-III), Simple Reaction Time, Finger tapping, Trailmaking A and B, the Stroop test, and Wisconsin Card Sort. The validity and rationale underlying the use of these tests in the clinical trial have been described (Martins et al., 2005).

Table 1
Neurobehavioral tests assessed with mean scores for Year 7 (final year of clinical trial).

Test/domain	Measure ^a	Boys, N=121 Mean (SD)	Girls, N=118 Mean (SD)
<i>Attention</i>			
Stroop Test–Color, Word	# correct ↑	66.41 (11.96)	69.23 (10.43)
Color–Word	# correct ↑	90.07 (15.18)	91.47 (15.23)
WAIS-III – Digit Span	# correct ↑	41.84 (9.78)	43.88 (8.75)
WMS III – Spatial Span	# correct ↑	14.34 (3.68)	14.14 (2.77)
Adult Trails A	# correct ↑	15.84 (3.02)	15.58 (3.12)
	Time (sec) ↓	26.35 (10.62)	30.38 (11.38)
<i>Visual–Spatial</i>			
Simple Reaction Time	Time (sec) ↓	0.74 (0.15)	0.77 (0.13)
WAIS III – Digit Symbol	# correct ↑	72.45 (17.09)	76.68 (13.87)
Symbol Search	# correct ↑	33.15 (8.85)	34.57 (8.04)
<i>Executive Functioning</i>			
Wisconsin Card Sort – categories completed	# categories ↑	3.06 (1.38)	3.08 (1.47)
Adult Trails B	Time (sec) ↓	65.69 (26.99)	63.41 (23.67)
<i>Learning and Memory</i>			
RAVALT Tr1 – List A	# correct ↑	5.61 (1.49)	6.10 (1.86)
Tr5 – List A	# correct ↑	11.24 (2.20)	11.54 (2.23)
Tr6 – List B	# correct ↑	4.73 (1.38)	5.27 (1.56)
Tr7 – List A/Post B	# correct ↑	9.85 (2.56)	10.22 (2.48)
Tr8 – List A after 20'	# correct ↑	9.30 (2.72)	10.07 (2.77)
WMS-III – Visual Reproductions – Immediate	# correct ↑	34.72 (4.65)	36.61 (2.98)
Delayed	# correct ↑	31.98 (6.96)	34.90 (4.02)
CVMT d-Prime	Score ↑	1.51 (.94)	1.61 (.88)
<i>Motor</i>			
WRAVMA – Pegs – Dominant	# Pegs ↑	47.40 (8.50)	49.92 (6.30)
Non-Dominant	# Pegs ↑	44.33 (7.57)	45.02 (6.12)
Finger Tapping – Dominant	# Taps ↑	52.75 (5.59)	48.44 (5.72)
Non-Dominant	# Taps ↑	46.66 (5.90)	42.53 (5.81)

^a Arrows show direction of improved performance.

Table 1 lists the 23 neurobehavioral tests that were assessed and presents their means and standard deviations at their last year of administration (Year 7). Tests are organized within the behavioral domains that were evaluated in the clinical trial (DeRouen et al., 2006). Arrows depict whether the test score increases or decreases in magnitude with improved performance. Diminished or adversely affected performance associated with Hg exposure or CPOX4 variant status is described as occurring in the direction of impaired performance, whereas enhanced or beneficially affected performance associated with either of these variables is described as occurring in the direction of improved performance. The Comprehensive Test of Non-verbal Intelligence (CTONI) was given to each child at the beginning of the clinical trial to obtain a measure of IQ at baseline.

2.3. Genotyping assays

Genotyping for the present study was performed on DNA extracted from buccal cell samples that were obtained from study subjects following completion of the clinical trial ($n = 199$) or from blood samples that were obtained at baseline for blood lead assessments ($n = 152$). Genotyping was performed by the Functional Genomics Laboratory of the NIEHS Center for Ecogenetics and Environmental Health at the University of Washington, using automated DNA sequencing assays. Oligonucleotides used for PCR and sequencing as well as primer and allele-specific probes used for fluorescent 5'-nuclease assays have been previously described in detail (Woods et al., 2005). In the present study, each child was evaluated for CPOX4 gene status, and categorized as CPOX wild type (A/A) or CPOX4 (A/C or C/C) if either a single or double allelic variant, respectively, was found. Children were also characterized for the CPOX5 variant as CPOX wildtype (G/G) or CPOX5 (G/A or A/A) if either a single or double allelic variant, respectively, was found.

2.4. Human subjects considerations

All parents or guardians of children who participated in the clinical trial gave written consent, and all children provided signed assent, for the treatments and assessments made during the course of the trial, including collection of blood samples. Written consent was also obtained from all participants who provided buccal cell samples for genotyping subsequent to completion of the clinical trial. The study protocols for both the clinical trial and the present genotyping study were approved by the institutional review boards at the University of Lisbon and the University of Washington.

2.5. Urinary mercury analysis

A urine sample (~50 ml) was collected from each child at baseline of the clinical trial and at each subsequently scheduled annual visit to the University of Lisbon School of Dental Medicine for dental, neurologic, and neurobehavioral evaluations. Analysis of total mercury (Hg) was performed by continuous flow, cold vapor spectrophotometry, as previously described (Pingree et al., 2001). Urinary creatinine concentrations were measured using a standard colorimetric procedure (Sigma #555-A; Sigma-Aldrich, St. Louis, MO, USA). Urinary Hg concentrations were calculated as micrograms per gram creatinine ($\mu\text{g/g}$ creatinine). Urinary Hg concentrations were transformed into natural logs after adding one [$\ln(\text{HgU} + 1)$] and used in this form as a quantitative measure of Hg exposure for all statistical analyses.

2.6. Statistical analyses

This study evaluated whether CPOX4 or CPOX5 gene status affected the relationship between Hg exposure and tests of neurobehavioral functions among children who were evaluated annually from baseline

through 7 years of follow-up after initial placement of dental amalgam (Hg) or composite resin tooth fillings (DeRouen et al., 2006).

Urinary Hg concentrations (HgU) measured at each annual behavioral test session were employed as the measure of Hg exposure instead of the dichotomous assignment to amalgam or composite resin treatment groups as performed in the clinical trial. Treatment assignment from the clinical trial was evenly split among boys (81 from the composite group and 83 from the amalgam group), whereas girls came slightly more frequently from the amalgam group (74 composite and 92 amalgam). However, treatment assignment accounted, at most, for only 17% of the variation in HgU among boys (Year 2 $r^2 = 0.171$) and 15% among girls (Year 2 $r^2 = 0.154$).

Statistical analyses were performed using SPSS Version 19 (IBM®SPSS®, Chicago, IL, USA). The wide range in ages of subjects at the beginning of the clinical trial (8–12 years) and the changing of specific tests administered to various age groups during the course of the trial, e.g., child versus adult versions of some tests, militated against the propitious use of repeated measures analysis. Therefore, we employed concurrent urinary Hg concentrations (HgU) and neurobehavioral test performance data acquired from the second year of follow-up (Year 2, where mean HgU reached a peak among both boys and girls in the cohort) to estimate the *acute* effects of Hg exposure on performance, and both *maximum* and *cumulative* measures of HgU over the entire study period as well as performance outcomes during the last year of the study (Year 7) to evaluate *chronic* effects of Hg exposure.

Acute measures of Hg exposure were calculated as the natural log of HgU adjusted by 1 ($\ln[\text{HgU} + 1]$). The natural log best accommodates how exposures are distributed biologically, whereas adding 1 minimizes the influence of very small changes in HgU at very low levels (which could have otherwise dominated the analyses). *Maximum* HgU was simply the largest value of this *acute* measure across all study years. *Cumulative* HgU was calculated as the natural log of the sum of all HgU with 1 added to this summation ($\ln[(\sum \text{HgU}) + 1]$).

Because the effect of the CPOX4 variant on neurobehavioral performance as affected by Hg exposure was the principal focus of this study, we developed an analytical protocol reflecting this focus. As a *first step*, we used a base model that included the measure of Hg exposure (as defined above), CPOX allelic status (dichotomous as either wildtype or het/mut for CPOX4), and their interaction term. In addition, this model included the covariates of age at assessment, race, and non-verbal IQ (determined at baseline). Performances on neurobehavioral tests were each individually evaluated as the outcome variable in separate analyses for boys and girls.

Step two was initiated whenever there was a significant interaction term between Hg exposure and CPOX allelic status. This finding was taken as evidence of effect modification, and thus analyses of effect between Hg exposure and test performance were calculated separately among boys or girls genotyped as either CPOX wildtype or CPOX4 variant status. This strategy provided a clear description of Hg dose–response relationships within each genotypic group.

Step three involved evaluating main effects when the interaction term between Hg exposure and CPOX allelic status when genotyped for CPOX4, was *not* significant. In this case, the interaction term was dropped from the baseline model, and the main effects of Hg exposure and CPOX status were evaluated among the full cohort of boys or girls.

This analytical approach was repeated for children genotyped for CPOX5 allelic status.

Prior to fitting the regression models, we examined the assumptions of the model by scrutinizing the distributions and variances of all cognitive tests and $\ln(\text{Hg})$. Most distributions had no significant deviation from normality or inflated variance. After fitting each model, we examined the standardized residuals for statistical outliers. In the rare event that an outlier was found, it was removed and the model was refit.

3. Results

The study cohort consisted of 330 children (164 boys and 166 girls) for whom CPOX4 or CPOX5 gene status was available from among 507 total subjects enrolled at the start of the clinical trial. Excluded subjects either did not provide a blood sample at the initiation of the study, or were lost to follow-up for acquisition of a buccal cell sample following completion of the trial. Table 2 presents the characteristics of the cohort at Entry as well as at Years 2 and 7 of the clinical trial. Both boys and girls averaged 10.1 years of age, and most were in the 4th grade at entry into the study. Approximately 74% of boys and 71% girls were Caucasian at Entry, and each had an average non-verbal IQ score of 86. Table 2 also displays the mean “Raw” values for urinary Hg concentrations (HgU) and the natural log calculations for the HgU at Entry, Year 2 and Year 7 of the clinical trial, as well as the calculated *maximum* and *cumulative* values for both sexes at Year 7. Minimum and maximum urinary mercury concentrations for each entry are also presented. The change in number of total subjects between Entry and Year 7 reflects the overall 14% loss to follow-up over the course of the clinical trial (DeRouen et al., 2006). The frequencies of the homozygous common (wildtype), heterozygous, and homozygous mutant alleles for boys and girls for CPOX4 and CPOX5 are also presented.

3.1. CPOX4 analyses: effects on acute Hg exposure

Results of analyses of modification by the CPOX4 variant on neurobehavioral test outcomes associated with *acute* Hg exposure (based on HgU at Year 2) are presented in Table 3. Among boys, significant interaction effects between CPOX4 and Hg exposure were observed for 3 of the 23 tests of neurobehavioral function evaluated during the clinical trial. These included the Stroop color and word/color tests, both within the Attention domain, and dominant hand finger tapping, a test of Motor function. The significant dose–response relationships between performance and *acute* Hg exposure for these tests were all in the expected (adverse) direction and were restricted to

boys with the CPOX4 variant. There were no main effects observed for any of the other tests.

Among girls, CPOX4 significantly modified the effects of *acute* Hg exposure on only 2 tests of neurobehavioral function, the RAVALT Trial 5 and Trail 8–List 20', both within the Learning and Memory domain (Table 3). In both cases, significantly impaired performance associated with *acute* Hg exposure was observed only among girls with the CPOX4 variant. Also among girls, 4 tests were observed to have significant main effects associated with *acute* Hg exposure (not shown). Among these, only the Trails A test ($p \leq 0.03$) was in the impaired direction, while 3 tests, Stroop Color/Word ($p \leq 0.03$), RAVALT Trial 6 ($p \leq 0.02$) and the Peg test for the dominant hand ($p \leq 0.03$), had significant associations in the direction of improved performance. In addition, 2 tests, finger tapping dominant ($p \leq 0.001$) and non-dominant ($p \leq 0.01$), had significantly improved performance among girls identified as having the CPOX4 variant. Aside from Motor function, no domain of neurobehavioral function was found to have more than 1 test that achieved statistical significance in terms of either main effect of *acute* Hg exposure or CPOX4 gene status among girls. Therefore, no domain could be said to be predominantly affected by *acute* Hg exposure or CPOX4 variant status among girls.

3.2. CPOX4 analyses: effects on chronic Hg exposure among boys

In contrast to effects with *acute* Hg exposure, analysis of the effects of CPOX4 on neurobehavioral performance associated with *chronic* Hg exposure among boys showed numerous significant interaction terms across a wide range of tests and performance domains (Attention, Visual–Spatial, Executive Function, Learning and Memory, and Motor). These results are presented in Table 4. When *cumulative* exposure was used as the *chronic* Hg exposure measure, significant Hg dose–response effects were observed on 11 of 23 neurobehavioral test outcomes among boys with the CPOX4 variant, all in the impaired direction, with 7 associations being significant at $p \leq 0.01$. In contrast, no significant

Table 2
Study population characteristics for participants at Entry (baseline) and in Year 2 and Year 7.

Characteristics	Boys			Girls		
	Entry	Year 2	Year 7	Entry	Year 2	Year 7
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Age	10.15 (.84)	12.20 (.84)	17.14 (.86)	10.10 (.92)	12.16 (.95)	17.08 (1.02)
School Year	4.04 (1.05)	5.78 (1.2)	9.40 (2.04)	4.15 (1.07)	5.92 (1.16)	9.86 (1.52)
Non-Verbal IQ (at entry only)	86.26 (10.37)	–	–	86.26 (10.37)	–	–
Urinary Mercury Concentrations						
Raw HgU ^a	1.65 (1.25)	2.17 (2.02)	1.25 (3.00)	1.98 (2.40)	2.86 (2.63)	1.77 (2.27)
Range	(0.14, 7.61)	(0.05/11.5)	(.005, 31.7)	(0.11, 23.5)	(.007, 15.6)	(.006, 15.3)
Calculated Ln HgU ^b	0.89 (0.41)	1.02 (0.49)	0.62 (0.48)	0.94 (0.48)	1.18 (0.57)	0.83 (0.56)
Range	(0.13, 2.15)	(0.04/2.53)	(0.00, 3.49)	(0.10, 3.20)	(0.01, 2.81)	(0.01, 2.79)
Calculated Maximum ^b	–	–	1.46 (0.52)	–	–	1.68 (0.54)
Range	–	–	(1.32, 4.03)	–	–	(1.44, 4.13)
Calculated Cumulative ^c	–	–	2.47 (0.50)	–	–	2.74 (0.55)
Range	–	–	(0.53, 3.49)	–	–	(0.61, 3.20)
Distribution	% (N)	% (N)	% (N)	% (N)	% (N)	% (N)
Total Subjects (N)	164	160	121	166	151	118
Caucasian, % (N)	74.4% (122)	74.4% (119)	71.9% (87)	71.1% (118)	68.9% (104)	69.5% (82)
CPOX4						
Wildtype (A/A)	71.3% (117)	71.3% (114)	67.8% (82)	65.1% (108)	64.2% (97)	61.9% (73)
Heterozygous (A/C)	28.0% (46)	28.1% (45)	31.4% (38)	27.7% (46)	27.8% (42)	28.8% (34)
Homozygous Mutant (C/C)	0.6% (1)	0.6% (1)	0.8% (1)	7.2% (12)	7.9% (12)	9.3% (11)
CPOX5						
Wildtype (A/A)	54.3% (89)	53.8% (86)	54.2% (65)	61.7% (103)	62.5% (95)	64.7% (77)
Heterozygous (A/C)	37.8% (62)	38.1% (61)	38.3% (46)	32.9% (55)	32.9% (50)	31.1% (37)
Homozygous Mutant (C/C)	7.9% (13)	8.1% (13)	7.5% (9)	5.4% (9)	4.6% (7)	4.2% (5)

^a $\mu\text{g/g}$ creatinine.

^b $\ln(\mu\text{g/g creatinine} + 1)$.

^c $\ln[(\sum \mu\text{g/g creatinine}) + 1]$.

Table 3
Acute Hg dose–response effects among boys and girls with CPOX WT or CPOX4 variant in Year 2.

Measure	WT		Het or Mut	
	Beta (SE)	r _{part} (p)	Beta (SE)	r _{part} (p)
<i>Boys</i>				
Attention Domain				
Stroop Test – Color	–.19 (1.80)	–.01 (.92)	–8.40 (2.80)	–.42 (.005)
Stroop Test – Word/Color	1.09 (1.51)	.07 (.47)	–3.50 (1.68)	–.31 (.04)
Motor Domain				
Finger Tapping – Dominant	1.21 (.91)	.13 (.19)	–3.25 (1.53)	–.32 (.04)
<i>Girls</i>				
Learning and Memory Domain				
RAVALT TR 5	.28 (.43)	.07 (.51)	–1.34 (.47)	–.38 (.006)
RAVALT TR 8 – List A 20'	.34 (.45)	.08 (.46)	–1.69 (.55)	–.40 (.003)

Values in **bold** signify $p \leq 0.05$.

cumulative Hg dose–response effects on neurobehavioral test performance were observed among boys genotyped as CPOX wildtype.

Using maximum exposure as the chronic Hg exposure matrix, 14 tests demonstrated significant dose–response relationships among boys with CPOX4 variant status, 12 being significant at $p \leq 0.01$. The 15th test (WRAVMA Pegs Non-Dominant) reached near significance ($p \leq 0.06$). All of these effects are significantly adversely affected in relation to Hg exposure among boys with CPOX4 variant status. In contrast, among boys genotyped as CPOX wildtype, only 1 neurobehavioral function (Finger tapping-Dominant) was significantly associated with maximum Hg exposure ($p \leq 0.03$), and this was in the direction of improved performance (Table 4).

Table 5 presents the significant main effect relationships for tests of neurobehavioral performance for which significant interaction terms were not observed among boys in the present analyses. When evaluated in terms of the cumulative chronic Hg exposure matrix, 4 tests of neurobehavioral performance were significantly associated with Hg exposure and 1 specifically with CPOX4 gene status. While all of these associations were in the direction of impaired performance, most were of only borderline significance in terms of

suggesting independent effects of either chronic Hg exposure or CPOX4 gene status on neurobehavioral performance. When evaluated in terms of the maximum chronic Hg exposure matrix, 3 test outcomes were found to be significantly associated with Hg exposure, but none specifically with the CPOX4 variant.

3.3. CPOX4 analyses: effects on chronic Hg exposure among girls

In contrast to findings among boys, no significant dose–response relationships for Hg exposure were observed for either maximum or cumulative chronic Hg exposure measures when evaluated separately among CPOX wildtype or CPOX4 variant girls, despite the fact that 2 tests (WAIS-III Digit Span and RAVLT Trial 6–List B) had significant interaction terms between CPOX4 and maximum chronic exposure. Main effects analyses among all girls found no significant associations with either measure of chronic Hg exposure. However, 2 tests (finger tapping dominant and simple reaction time) had significant ($p \leq 0.01$ or $p \leq 0.02$) associations with CPOX4 for both chronic Hg measures. Three of these 4 associations showed significantly improved performance

Table 4
Chronic Hg dose–response effects among boys with CPOX WT or CPOX4 variant in Year 7.

Behavioral Test	N	Cumulative Hg				Maximum Hg			
		WT		HET OR MUT		WT		HET OR MUT	
		Beta (SE)	r _{part} (p)	Beta (SE)	r _{part} (p)	Beta (SE)	r _{part} (p)	Beta (SE)	r _{part} (p)
ATTENTION									
Stroop Test - Color	121	–1.14 (2.32)	–.06 (.63)	–12.55 (4.05)	–.47 (.004)	.73 (2.24)	.04 (.74)	–11.17 (3.80)	–.45 (.006)
Word	121	–2.90 (3.20)	–.10 (.37)	–18.06 (4.89)	–.54 (.001)	–.77 (3.10)	–.03 (.80)	–17.10 (4.51)	–.54 (.001)
WAIS-III - Digit Span	121	.11 (.77)	.02 (.88)	–3.57 (1.12)	–.48 (.003)	.87 (.74)	.13 (.24)	–3.90 (.98)	–.57 (.0001)
WMS-III - Spatial Span	121	.17 (.62)	.03 (.79)	–2.45 (.97)	–.40 (.02)	.57 (.60)	.11 (.35)	–2.89 (.85)	–.50 (.002)
Adult Trails A - Time (Sec)	120	–.80 (2.07)	–.04 (.70)	11.25 (3.83)	.45 (.006)	–2.37 (1.98)	–.14 (.24)	13.04 (3.30)	.56 (.0001)
VISUAL-SPATIAL									
SRT Mean	121	.00 (.03)	.01 (.96)	.15 (.05)	.44 (.007)	.01 (.03)	.02 (.86)	.14 (.05)	.46 (.005)
WAIS-III - Digit Symbol	120	–2.46 (3.33)	–.08 (.46)	–20.70 (6.08)	–.50 (.002)	–.32 (3.32)	–.01 (.92)	–18.17 (5.75)	–.48 (.003)
EXECUTIVE FUNCTION									
Adult Trails B	120	–	–	–	–	1.98 (6.08)	.04 (.75)	22.26 (7.03)	.48 (.003)
LEARNING & MEMORY									
RAVALT TR5 - List A	121	–	–	–	–	.51 (.43)	.13 (.24)	–1.78 (.80)	–.36 (.03)
TR7 - A/ post B	120	–	–	–	–	.52 (.52)	.11 (.33)	–2.24 (.82)	–.42 (.01)
TR8 - List A 20'	120	.32 (.60)	.06 (.60)	–2.01 (.92)	–.35 (.04)	.84 (.58)	.16 (.15)	–2.14 (.83)	–.40 (.01)
MOTOR									
WRAVMA – Pegs Dominant	121	–	–	–	–	–.55 (1.58)	–.04 (.73)	–7.16 (2.90)	–.39 (.02)
Non Dom	121	.41 (1.49)	.03 (.27)	–5.87 (2.92)	–.33 (.05)	.79 (1.44)	.06 (.58)	–5.34 (2.72)	–.32 (.06)
Finger Tapping - Dominant	119	1.59 (1.22)	.15 (.20)	–4.03 (1.76)	–.37 (.03)	2.59 (1.15)	.25 (.03)	–3.46 (1.65)	–.34 (.04)
Non-Dom	119	1.07 (1.27)	.10 (.40)	–7.24 (1.67)	–.60 (.0001)	2.14 (1.21)	.20 (.08)	–5.83 (1.66)	–.52 (.001)

Values in **bold** signify $p \leq 0.05$ in the direction of impaired performance. Highlighted value signifies $p \leq 0.05$ in the direction of improved performance.

Table 5
Significant main effects for chronic Hg exposure and CPOX4 among boys in Year 7.

Behavioral test	Cumulative Hg				Maximum Hg			
	Hg (ln)		CPOX4		Hg (ln)		CPOX4	
	Beta (SE)	r _{part} (p)	Beta (SE)	r _{part} (p)	Beta (SE)	r _{part} (p)	Beta (SE)	r _{part} (p)
<i>Attention</i>								
Stroop Test – Word/Color	–3.30 (1.64)	–.18 (.05)	–1.50 (1.78)	–.08 (.40)	–2.17 (.59)	–.13 (.16)	–1.64 (1.79)	–.08 (.36)
<i>Visual–Spatial</i>								
WAIS-III – Symbol Search	–3.48 (1.54)	–.20 (.03)	1.86 (1.67)	.10 (.27)	–2.91 (1.49)	–.18 (.05)	1.73 (1.68)	.10 (.30)
<i>Learning and Memory</i>								
WMS-III – Visual Reproductions – Immediate	–1.66 (.83)	–.18 (.05)	–.06 (.90)	–.01 (.95)	–1.63 (.80)	–.19 (.04)	–.11 (.90)	–.01 (.90)
Delayed	–2.95 (1.24)	–.22 (.02)	.62 (1.34)	.04 (.64)	–2.70 (1.19)	–.21 (.02)	.52 (1.34)	.04 (.70)
<i>MOTOR</i>								
WRAVMA – Pegs Dominant	.41 (1.49)	.03 (.27)	–5.87 (2.92)	–.33 (.05)	–	–	–	–

Values in **bold** signify $p \leq 0.05$.

among girls identified as having the CPOX4 variant versus those with CPOX wildtype.

3.4. Analyses of CPOX4 Effects by Neurobehavioral Domain

3.4.1. Attention

The Attention domain includes 3 Stroop subtests, the Digit and Spatial Span tests (considered to be more related to Attention and rehearsal than to Memory), and the Trails A test. One of the Stroop tests, the Color/Word test, introduces discordance between the color spelled out and the color in which the word is written. This test requires what may be called “directed attention”, in that it includes areas of brain function other than those specifically associated with attention, and thus is not as direct a measure of simple attention as the other tests.

Among the CPOX4 acute effects analyses (Table 3), 2 of the Stroop subtests (Color and Color/Word) had significant dose–response associations among boys with the CPOX4 gene variant. The CPOX4 chronic effects analyses (Table 4) involved all of the Attention Domain tests except for the Stroop Color/Word sub-test. These analyses suggest that Attention is highly impacted by Hg among boys with CPOX4 variant status when evaluated in terms of either maximum or cumulative Hg exposure.

3.4.2. Visual–Spatial acuity

The Visual–Spatial domain tests include Simple Reaction Time (responding to a visual stimulus), Digit Symbol (coding a symbol with a digit), and Symbol Search (scanning to find a target symbols). As shown in Table 4, significant associations for tests of Visual–Spatial acuity were found for analyses employing measures of chronic but not acute Hg exposure among boys. Two tests, Simple Reaction Time and Digit Symbol, had significant dose–response associations with both maximum and cumulative chronic exposure matrices among boys genotyped as having CPOX4 variant but not CPOX wildtype status. Additionally, in the main effects analyses (Table 5), the Symbol Search test had significant associations with both chronic Hg exposure matrices. These analyses show that the Visual–Spatial domain can be impacted by Hg exposures within this genetic subgroup.

3.4.3. Executive Function

Executive Function tests included Wisconsin Card Sort (sorting cards by changing criteria) and Adult Trails B (following a trail of alternating numbers and alphabet letters). As shown in Table 4, CPOX4 was found to significantly modify the effect of Hg exposure on only the latter test of Executive Function among boys, and this effect was restricted to the analysis employing the maximum exposure matrix as a measure of chronic Hg exposure. This effect, however,

was in the direction of impaired performance and the association was highly significant ($p \leq 0.003$)

3.4.4. Learning and Memory

The Learning and Memory domain included 9 measures consisting of 5 sub-tests of the RAVLT (an auditory verbal learning test using a list of 15 words with distraction and delayed recall), 2 Visual Reproductions tests (redrawing a figure immediately and delayed), and the CVMT test (recalling which visual objects are repeated). Among the analyses assessing the effects of CPOX4 variant status on acute Hg exposure effects (Table 3), CPOX4 was found to significantly modify the adverse effects of acute Hg exposure on 2 of the RAVALT sub-tests (Trail 5 and Trail 8 – list A 20') among girls, constituting the principal positive observation regarding the interaction of CPOX4 and Hg exposure among girls in this study. No associations of gene status with indices of chronic Hg exposure among girls were observed.

In contrast, 3 RAVALT sub-tests were found to have significant dose–response associations with the maximum Hg chronic exposure matrix among boys with the CPOX4 variant (Table 4), and 1 also having a significant association with the cumulative Hg exposure matrix. In the main effects analyses (Table 5), both immediate and delayed Visual Reproductions tests were found to have significant independent associations with both chronic Hg exposure matrices.

3.4.5. Motor function

The Motor function domain tests include Finger Tapping (number of taps in a fixed time) and the Pegs test (number of dowels inserted into a hole in a fixed time). Both tests evaluate dominant and non-dominant hand performance separately. All 4 of these tests either approached significance or were significantly associated with the maximum exposure matrix among boys carrying the CPOX4 variant, with 1 association in the direction of improved performance among boys genotyped as CPOX wildtype (Table 4). There were 3 tests (excluding Peg dominant hand) related to the cumulative Hg exposure matrix among boys with the CPOX4 variant. Whereas most of the associations observed in this domain demonstrated only moderate significance levels, the interaction of CPOX4 and Hg exposure on the Finger Tapping non-dominant hand test was highly significant.

3.5. CPOX5 analyses

No significant interaction effects were observed for CPOX5 and any measure of Hg exposure among either boys or girls. Moreover, no main effects were observed for CPOX5.

3.6. Summary of Results

CPOX4 gene status was found to modify the adverse effects of chronic Hg exposure on a wide range of neurobehavioral performance test results among boys. All of these effects were in the direction of impaired performance and spanned all 5 neurobehavioral domains. Many of the observed associations were highly significant ($p \leq 0.01$). These highly consistent observed associations, affecting more than half the neurobehavioral tests evaluated, cannot be dismissed due to the large numbers of tests and exposures evaluated. If we assume that the outcomes for each of the 23 tests evaluated were totally independent of each other, the binomial probabilities of our findings for the two chronic measures would be $< 2 \times 10^{-12}$ for cumulative exposure and $< 2 \times 10^{-17}$ for maximum exposures. While there clearly are strong correlations between performances on various behavioral tests, the consistency of these results is nonetheless highly compelling.

Finally, the converse lack of associations between Hg exposure and CPOX wildtype status suggests that the CPOX4 variant may be important in mediating neurotoxic effects of Hg exposure in boys. The absence of associations when comparable analyses were conducted for the CPOX5 variant argues against these observations being due to some selection process.

4. Discussion

Numerous studies have proposed a component of genetic susceptibility to neurobehavioral disorders associated with mercury and other xenobiotic exposures (Braun et al., 2006; Gundacker et al., 2010; Engström et al., 2008; Suk and Collman, 1998), although the modifying effects of commonly expressed genetic variants on these associations are just beginning to be defined. This is the first study, to our knowledge, to describe a genetic polymorphism that modifies the effects of mercury exposure on a wide variety of neurobehavioral functions in children. Previous studies provided evidence of significant associations between Hg exposure and the CPOX4 variant on neurobehavioral functions in adult dental professionals (Echeverria et al., 2006), although observed joint effects in that study were found to be strictly additive in nature. The present findings of synergistic, i.e., more than additive, interactions between Hg and CPOX4 on numerous neurobehavioral functions are consistent with potentially heightened susceptibility of children to the adverse neurobehavioral effects of Hg specifically associated with the CPOX4 genetic variant.

The paucity of findings of independent effects of Hg exposure on tests of neurobehavioral function in this study provide some consistency with findings from the dental amalgam clinical trial (DeRouen et al., 2006), in which exposure to Hg from dental amalgam was found not to be associated with deficits in any tests of neurobehavioral performance among either boys or girls. However, when controlling for CPOX gene status as performed here, Hg exposure was strongly associated with diminished performance across a wide range of the same tests, among boys with the CPOX4 variant. Diminished performance was most predominantly observed in tests of Attention, suggesting possible impairment of attentional vitality and flexibility, e.g., ability to sustain attention or to shift between 2 sequences held in working memory (Echeverria et al., 2002). Significant interactions between Hg exposure and CPOX4 on tests of Learning and Memory and of Visual-Spatial acuity were also observed, suggesting possible decrements of verbal learning and memory as well as of perceptual cognition. Effects on tests of Motor function, including measures of manual coordination and fine motor speed, also appear to be adversely affected when evaluated within the context of chronic Hg exposure among boys with the CPOX4 variant. These findings have important public health implications, inasmuch as mean urinary mercury levels among boys in this study ranged from 1.4 (1.3–1.6) $\mu\text{g/g}$ creatinine at baseline to a

maximum of 2.2 (1.8–2.5) $\mu\text{g/g}$ creatinine at Year 2 of follow-up in the dental amalgam clinical trial. By comparison, geometric mean urinary mercury levels measured among a nationally representative sample of children 12–19 years of age acquired as part of the 2003–2004 U.S. National Health and Nutrition Examination Survey (Centers for Disease Control Prevention, 2007) were 0.358 (0.313–0.408) $\mu\text{g/g}$ creatinine. Although this value is substantially lower than those measured in the present study, the mean urinary Hg concentration in the 90th percentile of that sample was 1.59 (1.13–2.52) $\mu\text{g/g}$ creatinine, comparable to the range of Hg concentrations at which adverse neurologic effects of Hg were observed herein among boys with CPOX4. These observations suggest potential adverse neurobehavioral effects of Hg among boys with the CPOX4 variant who fall within the top 10% of subjects sampled within that survey for Hg exposure.

The mechanistic association of CPOX4 to neurobehavioral functions remains to be delineated, although potential alterations in physiological heme availability and/or heme-dependent processes associated with diminished CPOX4 activity may underlie this effect (Li and Woods, 2009). In this regard, heme is known to play a critical role as a signaling molecule in glutaminergic neuronal receptor processing and synapse development (Chernova et al., 2006; Sengupta et al., 2005), as well as in the regulation of serotonin (5-hydroxytryptamine) synthesis and signaling in the central nervous system (Litman and Correia, 1983, 1985). Disorders of both systems have been implicated as etiologic in a variety of neurodevelopmental and neurobehavioral disorders (Chernova et al., 2011; Chugani et al., 1999; Smith et al., 2012), and both could be amenable to disruption by heme deficiency during critical periods of neurological development in children, particularly in association with mercury exposure (Li and Woods, 2009). While these observations provide a scientific rationale for the diminished neurobehavioral performance observed here among boys with the CPOX4 variant and Hg exposure, further studies are required to define the specific mechanistic events underlying this association.

The absence of effects of CPOX5 on neurobehavioral functions when evaluated in relation to any measure of Hg⁰ exposure in this study suggests that the CPOX4 variant may act in a genotype-selective manner to mediate the adverse neurobehavioral effects of Hg exposure observed here. While the potential effects of CPOX5 on CPOX enzymatic activity, heme bioavailability, or processes affecting neurological function are not known, CPOX5 need not be viewed as incapable of affecting biological processes, inasmuch as synonymous SNPs are widely recognized as mediating changes in translation kinetics, protein folding and other factors that underlie a wide variety of neurological and other disorders in humans (Chamary et al., 2006; Duan et al., 2003; Komar, 2007). Moreover, the heterozygous and full mutant variants of CPOX5 were distributed quite differentially from those of CPOX4 within this cohort, only 7 subjects (2%) sharing both CPOX4 and CPOX5 variant status, militating against selection bias in terms of findings observed with respect to those with CPOX4. Further research analyzing multiple SNPs within the CPOX gene as well as others associated with heme-dependent neurotransmitter processing pathways is required to identify the mechanisms underlying the apparent selective effects of CPOX4 seen here.

Notable differences between boys and girls in the effects of Hg exposure and the CPOX4 variant on neurobehavioral test performance were observed in this study. Although Hg exposure from dental amalgam was comparable among boys and girls participating in the clinical trial (DeRouen et al., 2006), sex-related differences in Hg toxicokinetics that may afford greater Hg excretion and, consequently, lesser likelihood of Hg retention and accumulation in girls than boys may contribute to this effect (Woods et al., 2007). Numerous other factors that include genetic and hormonal differences affecting brain development, structure and function between boys and girls are also likely to contribute to the gender differences observed here (Gochfeld, 2007; Hines et al., 2010; Vahter et al., 2007a,b; Valentino et al., 2012). Differences in

detection sensitivity for CPOX4 between boys and girls in this study have a less clear explanation, although genetic factors underlying gender differences in numerous psychiatric and neurobehavioral disorders have been reported (Baca-Garcia et al., 2002; Gaub and Carlson, 1997; Harrison and Tunbridge, 2008; Samochowiec et al., 2004). The observation that neither Hg exposure nor CPOX4 alone substantially affected neurobehavioral performance in girls suggests that sex-related predisposition, in addition to differences in Hg toxicokinetics, affects susceptibility. Of note, measures of cognitive function and other behaviors not specifically related to reproduction are often sex-linked, accounting for substantial differences in response to many chemical agents, with subsequent expression in behavior (Weiss, 2002). In this respect, many classes of chemicals including dioxins and polychlorinated biphenyls (Weiss, 2002), metals (nickel arsenic, lead, cadmium, mercury) (Vahter et al., 2007a,b), pesticides (paraquat, dithiocarbamate, triazole fungicides) (Vahter et al., 2007a), cigarette smoke (Kelada et al., 2002); and various classes of drugs (Calabrese, 1985) are reported to differentially affect neurologic functions in males and females, both in humans and animal models (Vahter et al., 2007a; Bjorklund et al., 2007; Gochfeld, 2007). The sexually divergent responses to Hg exposure and genetic disposition observed in the present study highlight the importance of considering such differences in development of strategies aimed at risk assessment and prevention, especially in children.

In conclusion, the present studies demonstrate significant adverse effects on neurobehavioral functions associated with chronic Hg exposure and the CPOX4 genetic variant among children, with effects manifested predominantly among boys. These findings are the first to describe a genetic polymorphism that modifies the effects of Hg exposure on neurobehavioral functions in children, and suggest directions for future research to define mechanisms underlying differential sensitivity to mercury between boys and girls.

Conflict of interest statement

JSW serves on the scientific advisory board of Ecoeos. All other authors declare no conflicts of interest.

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