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A quantitative evaluation of brain dysfunction and body-burden of toxic metals

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Toxic metal exposure (e.g. Hg, Pb, As) exposure is known to induce significant adverse effects on human brain function. The aim this study was to assess toxic metal body-burden in relation to potential brain dysfunction in patients diagnosed with neurological disorders (NDs).

Material/Methods: The Liberty Institutional Review Board (Deland, FL) approved the present study. Quantitative, fractionated, random urinary porphyrin testing (μg/L) from the Clinical Laboratory Improvement Act/Amendment (CLIA)-approved Laboratory Corporation of America (LabCorp) and cortical perfusion index (CPi) values from single-photon-emission-computed-tomography (SPECT) brain scans were employed to evaluate a prospective cohort of qualifying patients with diagnosed NDs (n=52) presenting for medical care at an endocrinology practice in the Cincinnati, OH area.

Results: Patients with more severe in comparison to mild brain dysfunction had significant increases in the mean urinary concentration of uroporphyrins (uP), coproporphyrins I (cP I), and total cP (cP I + III), as well as a trend towards significantly increased mean urinary concentration of pentacarboxyporphyins (5cxP) and cP III. A significant positive correlation between Hg body-burden associated porphyrins (5cxP + cP I + cP III) and increased brain dysfunction was observed.

Conclusions: The present study associated brain dysfunction with Hg body-burden in a cohort of patients diagnosed with NDs, but the contributions of other heavy metals or genetic factors cannot be ruled-out. Additional studies should be conducted to evaluate the consistency of the present findings with examinations of other populations.

key words: **cognitive functions • functional brain imaging techniques • mercury • porphyrins**

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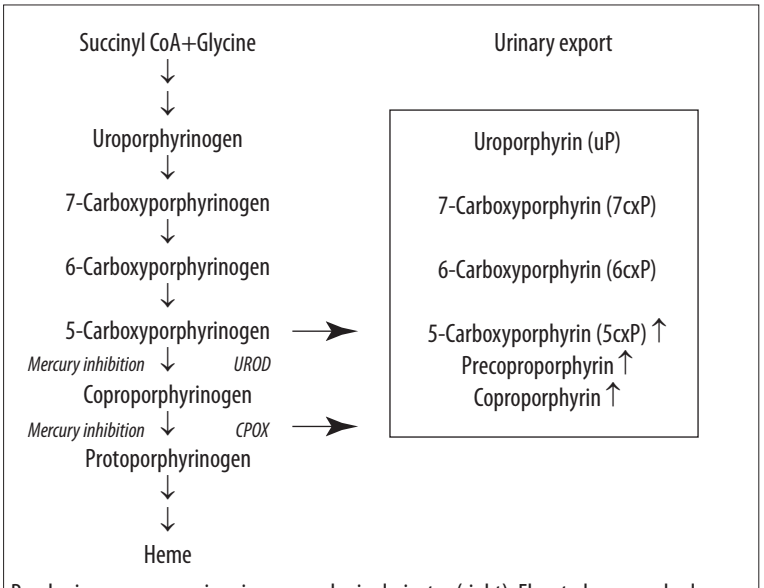
Background

Toxic metal exposure to mercury (Hg), lead (Pb), and arsenic As) can induce significant adverse effects on human brain function [1]. Among these metals, Hg in particular has been associated with significant deficits in measures of common neurological symptoms such as mood, short-term memory, tremor, and motor functions such as manual dexterity present in many adults patients diagnosed with neurological dysfunction. In addition, many Hg-exposed individuals were found to have significant increases in measurements of anxiety, schizoid symptoms, introvert behaviors, depression, aggression, and concentration [2,3].

Porphyrins are derivatives of the heme synthesis pathway that afford a measure of xenobiotic exposure [4,5]. Heme production primarily occurs in liver, kidneys, and erythroid cells by a synthetic process summarized in Figure 1 [6]. Excess porphyrinogen metabolites are excreted in the urine as oxidized porphyrins, particularly uroporphyrins (uP) and coproporphyrins (cP), the most abundant soluble porphyrin molecules in the kidney cortex [7]. Because these mid-pathway porphyrins are the most water-soluble of all the porphyrins, they are excreted predominantly in urine, whereas the hydrophobic protoporphyrin is predominantly found in the bile and feces. Excess urinary porphyrin excretion, or porphyrinuria, results from inhibition of key enzymatic steps in conditions including genetic deficiencies in heme production enzymes, hepatitis, renal, and erythroid disease as well as by heavy metal inhibition of heme enzyme synthesis [8]. The steps in the heme pathway most vulnerable to heavy metal inhibition are those involving uroporphyrin decarboxylase (UROD) and coproporphyrinogen oxidase (CPOX) [9]. The result of these inhibitions is specific elevations of cP in the urine.

A significant relationship between heavy metal inhibition of heme synthesis and porphyrinuria was demonstrated both in rats [10] and humans exposed to Hg [4] as well as in humans exposed to lead (Pb) [11]. Moreover, heavy metal removal with chelating agents reduced urinary porphyrin levels to control values [12]. Finally, a recent prospective, controlled human clinical trial revealed a significant dose-dependent relationship between increasing low-level exposure to Hg and increases in Hg-associated urinary porphyrins [13].

Among the various types of toxic metals, only potential neurological consequences of Hg intoxication have been evaluated by brain single-photon emission computed tomography (SPECT). For example, Hg neurotoxicity was assessed by examining regional cerebellar blood flow in patients with Hg-induced Minamata disease (MD) using technetium-99m ethyl cysteinate dimer (99m-Tc-ECD) [14]. These investigators performed SPECT on 15 patients with MD (eight men, seven women, aged 51–78 years, mean=70.5 years) and 11 control subjects (eight men, three women, aged 62–80 years, mean=72.5 years) and observed quantitatively significant reductions in 99m-Tc-ECD concentration in the inferior cerebellum among patients diagnosed with MD in comparison to the controls, even in the absence of significant brain atrophy on comparison MRI. Similarly, other investigators described a case-report of a patient with well documented inorganic Hg intoxication studied at the MRC Brain Metabolism Unit, Royal Edinburgh Hospital [15]. The patient was observed to have a brain SPECT scan that revealed significantly decreased posterior activity without significant brain atrophy observed by MRI. Most recently, Lin et al. found a dose-response relationship between cumulative mercury exposure index and specific uptake ratio in the striatum using brain SPECT [16].



Porphyrinogens appear in urine as porphyrin derivates (right). Elevated mercury body-burden can results in increased urinary 5-carboxyporphyrin, porphyrin, precoproporphyrin, and coproporphyrin by inhibiting uroporphyrinogen decarboxylase (UROD) and/or coproporphyrinogen oxidase (CPOX), urinary uroporphyrin is not reported to alter with inhibition of these enzymatic steps.

Figure 1. A summary of the heme synthesis pathway and major urinary metabolites.

Table 1. A demographic summary of the patients with neurological disorders examined in the present study.

Descriptive Information	Overall (n=52)	Mild[6] patients (n=26)	Severe[7] patients (n=26)	P-value8
Sex/age				
Female/male (ratio)	36/16 (2.25:1)	19/7 (2.7:1)	17/9 (1.9:1)	NS[9]
Mean age in years ±Std (range)	51±16.4 (19–84)	46±14 (19–79)	56±17.3 (24–84)	<0.05[10]
Race (n)				
Caucasian	73.1% (38)	84.6% (22)	61.5% (16)	NS[9]
Minorities[1]	26.9% (14)	15.4% (4)	38.5% (10)	
Urinary porphyrins (µg/L) [median][2]				
uP (lab reference range= 0–20)[3]	9.7±11.4 [8]	9.3±15.1 [5]	10.3±5.9 [9.5]	<0.01[10]
7cxP (lab reference range = 0–2)	4.2±9.5 [3]	5.7±13.3 [3]	3±2.2 [3.5]	NS[10]
6cxP (lab reference range = 0–1)	0.039±0.19 [0]	0.0±0.0 [0]	0.077±0.27 [0]	NS[10]
5cxP (lab reference range = 0–2)	1.4±6.7 [0]	0.54±1.8 [0]	2.3±9.3 [0]	0.08[10]
cP I (lab reference range = 0–15)	15.9±12.2 [13.5]	12.9±12.9 [8]	19.2±10.8 [17]	<0.005[10]
cP III (lab reference range = 0–49)	36.6±40 [23.5]	32.2±35 [19]	43.4±44 [31]	0.10[10]
Total cP (cP I + cP III)	52.5±50.5 [39.5]	45.1±47 [31]	62.5±52.8 [46]	<0.05[10]
SPECT Scan (%)[4]				
CPi (normal reference range = 55–78)[5] (range)	51.7±8.1 (31.8–70.0)	57.8±4.0 (53.2–70.0)	45.6±6.3 (31.8–52.8)	<0.001[10]
Median time between scan and porphyrin testing	7 Days	7 Days	7.5 Days	NS[10]

uP – uroporphyrins; 7cxP – 7-carboxyporphyrin; 6cxP – 6-carboxyporphyrin; 5cxP – 5-carboxyporphyrin; cP – Coproporphyrin; Std – standard deviation; NS – not significant; CPi – cortical perfusion index.

[1] Includes participants of Hispanic, Black, Asian, or Mixed Ancestry; [2] Mean ± standard deviation. Urinary porphyrins were measured in µg/L by LabCorp (CLIA-certified) blinded as to the diagnosis/clinical severity of the patients. Patients with uroporphyrin levels <2 µg/L (n=6) were excluded from the present study; [3] Mean ±2 standard deviations; [4] Mean ± standard deviation. SPECT CPi indices evaluated blinded to LabCorp urinary porphyrins; [5] Normal Refernce range is mean ±1 standard deviation; [6] Mild is defined as any study patient with a CPi SPECT score less than the overall study patient median (CPi score <53); [7] Severe is defined as any study patient with a CPi SPECT score greater than the overall study patient median (CPi score >53); [8] A comparison between the values for mild and severe patients; [9] The χ^2 test statistic was utilized (two-tailed); [10] The unpaired non-parametric Mann Whitney U test statistic was utilized (two-tailed).

The purpose of the present study is to integrate the combined technologies of urinary porphyrin testing and brain SPECT scanning to evaluate the relationship between toxic metal body-burden and brain dysfunction in a cohort of consecutive patients with diagnosed neurological disorders presenting for medical care at an endocrinology practice.

Material and Methods

The present study received Institutional Review Board (IRB) approval from the Liberty IRB (Deland, FL).

Patients examined

The present prospective study examined consecutive qualifying patients diagnosed with neurological disorders from the Cincinnati, Ohio area among patients presenting for routine endocrinology evaluation or brain SPECT referral for neurological complaints. Patients examined in the present study had basic demographic and medical diagnosis information collected at the time of initial presentation for inclusion in the present study. Table 1 summarizes the overall demographic features of the cohort of patients examined in this study. Patients with a concurrent diabetes mellitus diagnosis were excluded from analysis in the present study because no correlation of urinary porphyrin excretion with SPECT parameters in diabetics, possibly because of alteration in kidney function as well as baseline abnormalities in diabetics in responses to cerebral blood flow stimulation [17]. Table 2 summarizes the respective diagnoses of the patients examined in the present study.

Urinary porphyrin/SPECT testing

Urinary samples were collected from patients at the endocrinology practice and sent to the Clinical Laboratory

Table 2. A summary of the different types of diagnoses present among the patients with neurological disorders (n=52) examined in the present study.

Diagnosis type	Percentage of patients (n)*
Bipolar	9.6% (5)
Depression	25% (13)
Autism spectrum disorder	3.9% (2)
Memory loss	57.7% (30)
Anxiety disorder	17.3% (9)
Fibromyalgia	11.5% (6)
Parkinson's disease	5.8% (3)

* Patients may have one or more diagnoses.

Improvement Act/Amendment (CLIA)-approved Laboratory Corporation of America (LabCorp) for fractioned random urine porphyrin testing using methods of collection as defined by LabCorp. The lab used in the present study received no information regarding the results of SPECT testing on the patients examined. Patients' urine samples were tested for the following (measured in μg/L): uP, 7-carboxylporphyrins (7cxP), 6-carboxylporphyrins (6cxP), 5-carboxyporphyrins (5cxP), cP I, cP III, and total cP (cP I + cP III). Further, to help eliminate artificial dilution and ensure the validity of the specimens examined, patients with uP levels <2 μg/L were excluded from the present study (n=6).

The SPECT scanner used in most cases was a modified Park dual-head instrument with intrinsic Full-Width-Half-Maximal (FWHM) resolution of 2.5 mm and collimated system resolution of 8 mm at 140 keV and 12 mm at 511 keV. The camera sensitivity for 511 keV is 65 counts/min-μCi (0.375 inch detector camera) and 90 to 100 counts/min-μCi at 140 keV (0.375 inch NaI detectors, using 511 keV collimators). With doses of 8 mCi (18)F-2-deoxy-fluoro-D-glucose (FDG) (the 511 keV metabolism tracer) and 20 mCi Tc-99m-hexamethylpropylene amine oxime (Tc-99m-HMPAO), (the 140 keV perfusion tracer) the average cross talk of 140 keV to the 511 keV window ("upscatter") was less than 1% of the 140 keV counts and the average cross talk of 511 key to the 140 keV window ("downscatter") was less than 15% of the 140 keV counts. Downscatter was subtracted on a pixel by pixel basis prior to processing which typically used an order 6 Butterworth 0.35 filter for the 140 keV perfusion images and similar order 6 Bufterworth 0.27 filter for the 511 keV metabolism images. In addition, a single-head SPECT scanner manufactured by IS2 was also used a few cases. This IS2 instrument had initial sensitivity 190 cpm/μCi for Tc-99m with resolution 7 mm FWHM and by using an ultra high resolution collimator was modified to sensitivity 140 cpm/μCi for Tc-99m with resolution 5 mm FWHM. The same (Smartsoft) processing software was used with the IS2 as the Park SPECT scanners. Control studies with low disease-likelihood patients showed similar tracer distribution with the single-head IS2 instrument and the Park SPECT instruments. The IS2 SPECT performs dual acquisition with routine nuclear tracers; however, it does not have a high-energy collimator for 511 keV imaging.

For calculation of the Cortical Perfusion index (CPi), as defined below, a 9-point smooth of the perfusion-stimulated SPECT scan was followed by an iterative reconstruction (Park Medical Systems Inc.) produced values within 2% of those obtained for CPi in the same patients (n=5) when FDG was not present, verifying both the reproducibility of the CPi calculation and the removal of any FDG crosstalk for purposes of the CPi calculation. This procedure was verified for the entire range of CPi encountered, from approximately 15% to 80%. The imaging matrix was 128×128 with 5.36 mm (or 3.37 mm for the IS2 instrument) pixels and the SPECT scan used 2.8 degree (or 3.8 degree for the IS2) stops with 50 sec per stop and a total imaging time of approximately 40 min. Tracers were injected via an angiocatheter in a quiet room with the patient's eyes closed; the perfusion tracer was injected at least 15 minutes after 0.5 to 1.0 gram acetazolamide was administered by intravenous infusion (IV) or within 5 minutes of 0.4 mg to 0.8 mg nitroglycerin sublingual or at least 90 min after 100 mg cilostazol oral any of which produced similar cerebral perfusion stimuli in control studies. Minimal neurological stimulation was maintained during the uptake period of approximately 30 minutes prior to and during the SPECT scan. Control studies revealed minimal changes in scans beginning from 15 to 45 minutes after injection of the tracers. The CPi was calculated from ratios of computer-derived 60% (representing the cortical activity) divided by 30% (representing the whole brain activity) isocontour enclosed activities. The approximate normal range for CPi values (66.4±11.4%) was derived from studies of 40 patients with only minor neurological disease, none of whom had developed significant or even more severe abnormality within one to three years follow-up.

Regional values for CPi were normalized by multiplying the regional activity (instead of the 60% isocontour activity used in the numerator for usual CPi calculation) by the ratio of its area to the area of the 60% isocontour and then dividing by the activity of the 30% isocontour (the usual denominator for CPi calculation, so that regional CPi remains a dimensionless ratio, just like CPi). Regional values, for example a frontal or a parieto-occipital CPi, thus have nearly the same mean value as the CPi, but a slightly broader normal range depending mainly on the Poisson counting statistics in the smaller region. Control studies in which the same patients were studied with simultaneous or separate-day acquisitions verified reproducibility of these methods both for visual analysis of the images and the quantitative values of CPi, whose standard error varied by an average of less than 3% for studies performed within two weeks of each other on the same patient.

Statistical analysis

The current study used the statistical package contained in StatsDirect (Version 2.7.2). Urinary porphyrins measured at LabCorp between participants with mild brain dysfunction (CPi score < overall median value) in comparison to those with severe brain dysfunction (CPi score > overall median value) were evaluated utilizing the unpaired nonparametric Mann-Whitney *U*-test statistic. This division of data was undertaken because of the size of the sample examined, so as to ensure equal numbers of study participants in each group, as well as to provide adequate statistical power for

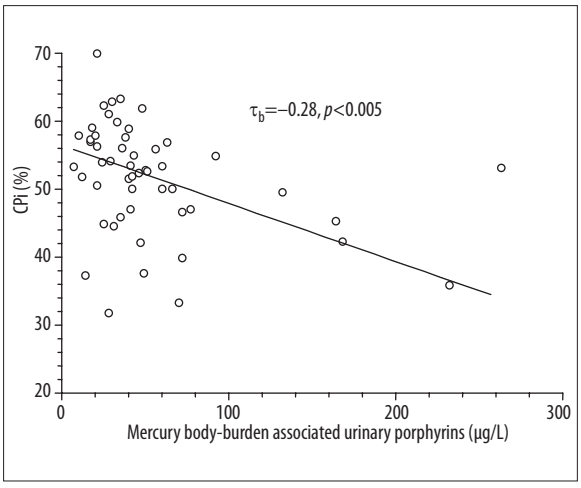


Figure 2. A summary of the correlation* between CPi values and urinary porphyrins. * The non-parametric linear sum regression test statistic was utilized (two-tailed). The mercury body-burden associated urinary porphyrins = 5cxP + cP I + cP III.

discerning statistically significant differences between the groups studied. The null hypothesis stated that there should be no difference between the overall distributions for each urinary porphyrin between participants with mild or severe brain dysfunction. The nonparametric linear sum regression test statistic was utilized to evaluate the relationship between urinary porphyrins-associated with Hg body-burden (5cxP + cP I + cP III) levels and CPi values for the study participants. The null hypothesis stated that the slope of the line would be equal to zero for the relationship between urinary porphyrins-associated with Hg body-burden and CPi values.

For all statistical tests in the present study, nonparametric testing was utilized to minimize potential assumption of normality for the data distributions examined, as well as to minimize the potential effects of outliers in the data examined. A two-tailed p value ⩽0.05 was considered statistically significant for all statistical tests. There was no correction made for investigation of multiple hypotheses.

Results

Table 1 summarizes urinary porphyrins among the patients diagnosed with neurological disorders who were examined in the present study. Among patients with severe brain dysfunction as measured using SPECT scanning in comparison to mild brain dysfunction, there were significant increases in the mean urinary concentration of uP (1.1-fold, p<0.01), cP I (1.5-fold, p<0.005) and total cP (1.4-fold, p<0.05). Further, there was a trend towards a significantly increased mean urinary concentration of 5cxP (4.3-fold, p=0.08) and cP III (1.3-fold, p=0.10). In addition, the other urinary porphyrins examined showed no significant differences among patients with severe brain dysfunction in comparison to mild brain dysfunction.

Figure 2 evaluates the correlation between SPECT scan generated CPi values and the urinary porphyrins examined. There was a significant positive correlation (τ_b=–0.28, p<0.005) between Hg body-burden associated porphyrins (5cxP + cP I + cP III) and decreasing CPi values using the nonparametric linear sum regression test statistic. In contrast, other urinary porphyrins that are not significantly associated with Hg body-burden (uP + 7cxP) showed no significant correlation with CPi values (data not shown).

Discussion

The present study provides the first quantitative evaluation of the relationship between heavy metal body-burden measured using urinary porphyrin testing and CPi as measured using SPECT scanning of the patients' brains. Using correlation and several Mann-Whitney tests, there was a significant correlation between increasing Hg-associated urinary porphyrins and worsening brain functioning. Further, using correlation and several Mann-Whitney tests revealed no consistent significant association between brain function and urinary porphyrins not associated with Hg body-burden.

In considering the context of the present findings, previous studies noted that distinct changes in urinary porphyrin concentrations were observed as early as 1–2 weeks after initiation of Hg exposure, and that these changes increased in a dose- and time-related fashion with the concentration of Hg in the kidney, one of the principal target organs of Hg compounds [8]. In addition, urinary porphyrin profiles were also shown to correlate significantly with both Hg body-burden and specific neurobehavioral deficits associated with low-level Hg exposure [8]. Furthermore, two recent studies evaluated urinary porphyrin metabolites in prospective, blinded cohort studies of subjects diagnosed with an autism spectrum disorder (ASD) [18,19]. These studies evaluated ASD severity based upon Childhood Autism Rating Scale (CARS) scores calculated prior to blind lab testing for urinary porphyrins. In comparison to study subjects with a mild ASD diagnosis, study subjects with a severe ASD diagnosis had significantly increased urinary porphyrin levels of 5cxP, prcP and total cP (I + III), whereas other urinary porphyrin levels were similar in both groups. In addition, regression analyses showed significant relationships between increasing CARS scores and rising urinary 5cxP, prcP, and total cP (I+III) levels. These correlations were absent for other urinary porphyrin metabolites examined. Finally, increasing urinary 5cxP and prcP levels were significantly correlated with impaired glutathione-dependent detoxification pathways.

Study limitations

In considering the potential limitations of the present study, there were no direct measurements of Hg levels in biological samples (i.e. brain, blood, urine, hair, etc.) taken from the patients under study. Instead, the present study examined a well-established biological pathway in urinary porphyrin testing as an indirect measurement to estimate Hg body-burden in the subjects examined. While it might be useful to measure Hg levels in the blood from the patients under study, these sources of measurement do have significant limitations. Specifically, it is well established that the half-life of free Hg in the body for at most several weeks after exposure, after which time, Hg is known to become lodged in tissues, especially the kidneys and brain [20], of the exposed individual, and it is the persistent Hg in human tissues that is associated with the greatest long-term

adverse consequences. As a result, since urinary porphyrins have been found to be an indirect measure of the level of Hg found in the tissue (i.e. the kidney), and since the increased urinary porphyrins observed occur as the result of physiological dysfunction, the present study has a significant advantage over more direct measurements of Hg in such biological samples as the blood because it indicates Hg tissue levels and these tissue levels produce the long-term adverse effects seen in humans.

In addition, another potential limitation of the present study is that non-metal agents can target the heme pathway and, as a result, elevate urinary porphyrin levels [21]. For example, other heavy metals such as Pb may have significantly contributed to the elevated cP levels [11], or genetic influences may have contributed to elevated porphyrins [22] observed in the present study. It is impossible to rule-out these phenomena, and they should be further evaluated in future studies. Despite this potential, specific urinary porphyrins known to be associated with increased Hg body-burden were consistently associated with the CPi measured-brain dysfunction by the statistical methods of analysis employed in the present study, whereas other urinary porphyrins, which are not associated with Hg body-burden but may be associated with non-metal agents, were not observed to correlate with the observed levels of brain dysfunction.

Another potential limitation of the present study was the moderate number of study participants. Given the moderate number of study participants in the present study, the brain dysfunction status of study participants was only divided into two groups (mild and severe). In a large study it may have been possible to divide the brain dysfunction status of study participants into more groups. In addition, in a large study it might be possible to further evaluate interactions between more variables.

It is also possible that the statistically significant relationships observed between urinary porphyrins and brain dysfunction might be the result of confounding or mere chance. In order to address the potential for confounding in the data examined, specific efforts were made in the present study to evaluate potential confounders such as gender, age, race, and time between urinary porphyrin testing and SPECT scanning when comparing patients with severe brain dysfunction in comparison to mild brain dysfunction. However, there were no significant differences in any of these potential confounders between the groups examined, except for age. Study participants with severe brain dysfunction were significantly older than patients with mild brain dysfunction, but the overall difference in mean ages was only 10 yrs and both groups had similar overall ranges for the ages of the subjects examined. It is unclear how the age difference observed could explain the significant differences observed in specific urinary porphyrins among those with severe neurological dysfunction in comparison to mild neurological dysfunction, apart from older age allowing for greater opportunity to be exposed to toxic levels of Hg. In addition, investigators have evaluated the specific effects of gender and sex on CPi values in brain SPECT imaging [23,24]. These studies described that cerebral perfusion, believed related to CPi values did not significantly differ between male and female subjects and was only weakly related or not related to age. Further, some investigators have even reported that stimulated cerebral perfusion measures, such as CPi, may actually be higher, not lower, in older subjects in comparison to younger subjects [25]. If such phenomena occurred in the present study, the result would be an underestimate of the true association between urinary porphyrins and brain dysfunction.

Further, the consecutive, blinded design of the present study helps to minimize potential confounding in the data. Namely, patients were examined that presented for endocrinology care in the Cincinnati, Ohio to a facility not actively engaged in recruiting patients for detoxification therapy for heavy metals, and those conducting the SPECT studies and the urinary porphyrin lab testing were blinded to one another's findings.

Another important limitation of the present study was there was no correction made for multiple statistical tests. It is possible that tests with p-values about 0.01 may be suspect, but in support of the results observed in the present study not being the result of mere statistical chance, the effects observed were found to be significant using non-parametric statistical tests, which make minimal assumptions about the overall distribution of the data examined. Further, because a limited number of statistical tests were conducted on the data examined in the present study, a two-sided p-value ≤ 0.05 was considered statistically significant, and many of the significant relationships observed had two-sided p-values <0.01, it is unlikely that the observed statistically significant relationships observed were the result of mere statistical chance.

Conclusions

The present study employed techniques previously not utilized in concert to evaluate the potential relationship between Hg body-burden and brain dysfunction. The techniques employed revealed a quantitative, significant relationship between increasing Hg body-burden and increasing brain dysfunction in a cohort of patients diagnosed with neurological disorders. A series of potential confounders were examined in the data but were found to have apparently limited effects on the significant relationships observed. It is possible that other heavy metals or genetic influences may play a role in the phenomena observed in the present study.

It is recommended that future studies further examine the relationship between heavy metal body-burden measured using urinary porphyrin testing and neurological dysfunction measured using brain SPECT scanning to determine CPi on an expanded cohort of patients with neurological disorders. Further, it is suggested that treatment protocols be designed to help ameliorate the apparent adverse effects of increased Hg body-burden among patients diagnosed with neurological disorders, and that the brains of these patients in these protocols be quantitatively evaluated for their level of brain dysfunction pre- and post-treatment using SPECT scanning techniques.

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Potential conflicts of interest

David Geier and Mark Geier have been involved in vaccine/biologic litigation. None of the authors of the present study have any financial interest in the labs utilized for testing.

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