Mixtures, Metals, Genes and Pathways: A Systematic Review

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ABSTRACT

Studies have shown associations between exposure to environmental concentrations of individual metals such as lead, arsenic, cadmium, and manganese and developmental outcomes in children, including reduced IQ and decreased performance on developmental tests. Studies focusing on potential mechanisms have identified potential pathways by which individual metals exert their effects, as well as genomic interactions influencing uptake rates. Given the action of individual metals, and the potential for similar cellular targets and activities, this systematic review evaluates the evidence for combined impacts of exposure, particularly under the assumption of additivity and pathways by which greater (or less) than additive effects are expected to occur. The review shows there is a significant body of evidence showing a greater than additive effect of combined exposures to lead, arsenic, and manganese, and to a lesser extent, cadmium. Potential pathways by which these metals interact include molecular initiating events leading to disruptions in neurotransmitter release, in cell signaling pathways, and by oxidative damage to neuronal cells. Pathways can be identified by which these cellular responses lead to decreased performance on a battery of animal and human developmental tests. Current risk assessment guidance calls for an individual chemical-by-chemical approach that fails to capture potential interactive effects of exposure to environmental mixtures combined with genomic influences, as have been documented here for several metals.

Keywords:

Arsenic, lead, manganese, cadmium, developmental, adverse outcome pathway, systematic review, integrated assessment

1.0 BACKGROUND

The regulatory science of risk assessment is used to inform decision making at scales ranging from site-specific remediation to broader policy development. Current risk assessment guidance dictates a largely chemical-by-chemical evaluation of exposures and risks which fails to adequately address potential interactions with other chemicals, non-chemical stressors, and genetic influences. As there are no standard approaches for developing toxicity values for use in risk assessment in the context of complex exposures and interactions, synthesis and integration across disciplines and individual studies focused on different aspects of the continuum from environmental fate – exposure – toxicology – health outcome are required to assess the likelihood of adverse effects occurring as shown in Figure 1. Typically, exposures to mixtures are assessed assuming either independence or additivity. Methods for developing regulatory values incorporating synergistic or antagonistic effects are not well-developed, although evidence for these kinds of interactions continues to grow.

The association between developmental health outcomes in children and exposure to either lead (Pb) (ATSDR 2007), manganese (Mn) (ATSDR 2007), arsenic (As) (ATSDR 2007) and cadmium (Cd) (ATSDR 2007) is well-established. In general, the assessment of developmental outcomes is non-specific (e.g., poor performance on IQ and other developmental tests) although evidence is growing of specific associations with Attention-Deficit Hyperactivity Disorder (ADHD) for both Mn (Schettler 2001) and Pb (Braun et al. 2006; Nigg et al. 2008) and autism () from epidemiologic studies. Evidence for the potential impact of combined exposures is less well understood (Wasserman et al. 2011; Kim et al. 2009; Wright et al. 2006) but continues to grow.

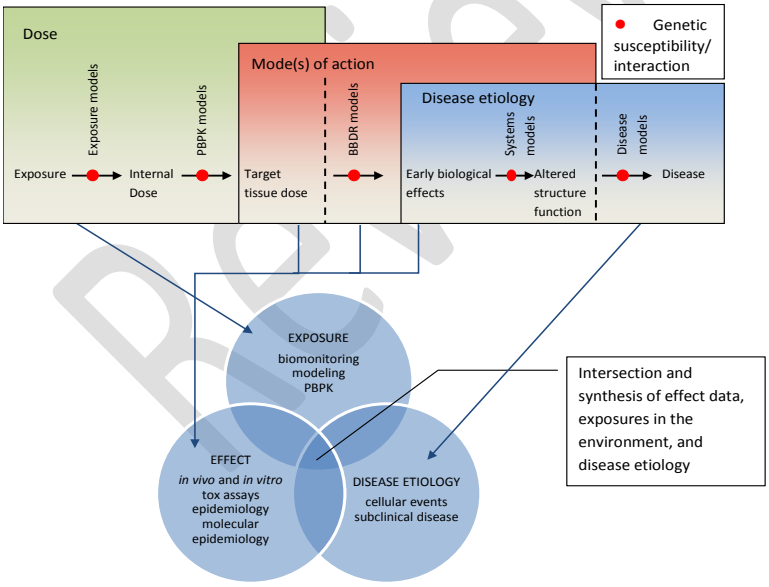

Figure 1: Exposure - Effect - Disease Continuum

Separately, the role that the genome might play in interacting with exposures to influence metal metabolism and uptake (Roy et al. 2013; Hopkins et al. 2008; Wang et al. 2007; Claus Henn et al. 2011)

and result in observed health outcomes (Gundacker et al. 2010; Nigg et al. 2010) provides additional data on factors contributing to variability in population response.

The Agency for Toxic Substances and Disease Registry (ATSDR) has developed "interaction profiles" for several chemicals including lead, cadmium, chromium, and arsenic (ATSDR 2004, Pohl et al. 2003) based on a binary weight-of-evidence approach (ATSDR 2001a, 2001b), providing conclusions regarding the expected direction of interaction for specific health outcomes and the degree of confidence in these conclusions. That assessment found evidence for a greater than additive effect of combined exposures to lead and arsenic for developmental outcomes, and a potentially greater than additive effect of combined exposures to Pb and Cd. An analysis for Cd and As was not presented. By definition, the binary weight-of-evidence approach only considers binary interactions. Nonetheless, that analysis provides evidence of a greater than additive effect of combined exposures to Pb and As and Pb and Cd and associations with developmental health outcomes. There are no published studies that extend the analysis to combinations of three and four constituents simultaneously.

Well-developed physiologically-based pharmacokinetic (PBPK) modeling approaches exist for Mn (Taylor et al. 2012; Teeguarden et al. 2007), As (Hays et al. 2010; El Masri and Kenyon 2008), Cd (Choudhury et al. 2001; Diamond et al. 2003; Kjellström and Nordberg 1978), and Pb (the Integrated Exposure UptakeBiokinetic [IEUBK] Model for Lead in Children, available from www.epa.gov/superfund/lead/products.htm). Again, there are no published studies that extend the analysis to mixtures of metals, although these kinds of PBPK approaches are being developed for other kinds of mixtures (Haddad et al. 1999; Haddad et al. 2001) and for predicting *in vivo* exposures associated with *in vitro* assay results (Yoon et al. 2013; Thomas et al. 2012; Wetmore et al. 2013), including the specific context of neurodevelopmental effects (Breier et al. 2008). Similarly, there are numerous studies exploring potential pathways by which these individual metals influence developmental outcomes, but no published studies for combinations of metals.

As a first step, we evaluate the potential for exposure to environmentally-relevant concentrations of mixtures of metals in conjunction with genetic influences to result in specific health outcomes through a critical evaluation of the intersection of environmental exposures (environmental exposure concentrations relative to biologically-effective doses), the evidence for particular effects from toxicological and epidemiological data, and what is known about cellular events at the subclinical level in relation to disease etiology to identify potential pathways and mechanisms. This allows an evaluation of

biological plausibility with respect to a hypothesized mode-of-action based on the best available understanding of molecular events required for disease progression, evaluated in the context of what is known about how these compounds exert their biological influence, and exposure conditions necessary to achieve absorbed doses relevant to the pathways of interest.

We explore these emerging issues in risk assessment in the context of exposures to mixtures of metals, including lead, arsenic, cadmium and manganese, and neurodevelopmental health outcomes in children. Included is a discussion of emerging methods for characterizing potential risks from exposure to mixtures together with potential gene-environment interactions (Liu et al. 2012; EC 2011; Sargiannis and Hansen 2012; Backhaus and Faust 2012). We provide a review of the literature on gene-environment and chemical mixture interactions, particularly in the context of neurodevelopmental health outcomes in children. We discuss examples of approaches for synthesizing exposure, effect, and disease etiology with an emphasis on our own research on exposure to mixtures of metals and neurodevelopmental effects at the center of the Superfund Research Program at the Harvard School of Public Health (Claus Henn et al. 2011; 2012; Bellinger 2012).

2.0 METHODS

We first conduct a literature search to develop a database of studies categorized by exposure, toxicology, epidemiology, methods, gene-environment, and health outcome and summarize the results with respect to the strength of the evidence for specific associations and potential mechanisms for the relationship between exposure to mixtures of metals and neurodevelopmental health outcomes. We evaluate the evidence for potential mechanisms by which interactive effects might occur using an adverse outcome pathway framework (OECD 2011; 2013) as shown in Figure 2.

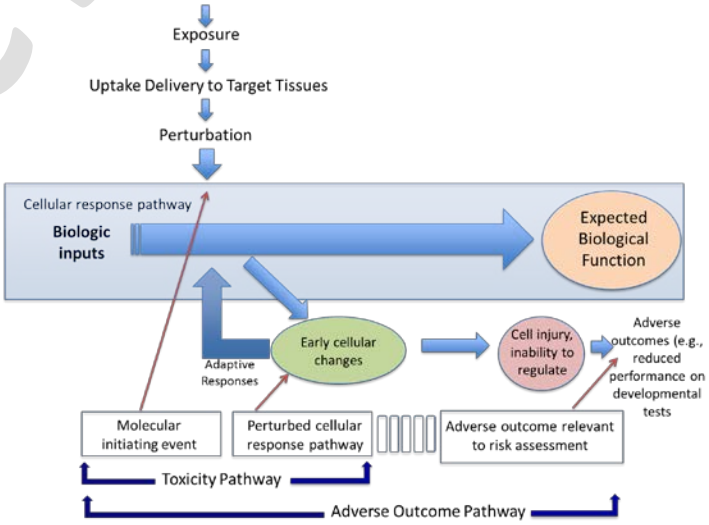

Figure 2: Schematic of the Adverse Outcome Pathway Continuum

2.1 Literature search strategy

The literature search relied on standard search engines including PubMed, Web of Science, Google Scholar, and other databases available through the Harvard University Library system. Search terms included all terms related to metals, mixtures, individual metals (e.g., arsenic, lead, manganese, cadmium), neurodevelopmental health outcomes, and associated MeSH terms. Reference lists for individual citations were reviewed to identify additional studies not identified through the literature search.

Another literature search involved the Comparative Toxicogenomics Database (available from www.ctdbase.org). The database was searched to identify 1) top interacting genes for each of the four metals, 2) genes in common to all four metals related to learning and cognitive disorders generally, including specific outcomes such as ADHD and autism, as well as non-specific outcomes, and, 3) all genes related to learning and cognition for each metal individually.

2.2 Database development

Each study was reviewed by one of the authors and relevant data and information extracted into a database that included the overall approach and study design, information on target tissues or organs, relevance to biological and molecular pathways, target genes (if applicable), exposures and study duration, and conclusions. Each study was categorized (e.g., epidemiologic, toxicological, exposure, review, outcome-based). Only those studies focused primarily on developmental or neurotoxic endpoints were included; those focused on carcinogenesis or other systemic effects were not included unless there was a particular relevance to a neurotoxic or developmental outcome.

We also explored overlapping gene targets using the Comparative Toxicogenomics Database (ctdbase.org). As an initial evaluation, we identified genetic targets in common across the metals with a particular emphasis on those genes with documented impacts on learning, development, cognition, and specific developmental outcomes such as ADHD and autism. For the genes identified from the comparative toxicogenomics database, a color-coded table was developed highlighting genes in common to all the metals, and a table of genes, gene function, and the impact of the individual chemical exposure (e.g., activation, inhibition) developed.

2.3 Analysis

Toxicological studies were first evaluated using a modified version of the Toxicological data Reliability Assessment Tool (ToxRTool) available from the European Commission Institute for Health and Consumer Protection at http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/archive-publications/toxrtool to determine initial data quality for each study. Each study was then further evaluated for evidence of a greater or less than additive effect with respect to the outcome being studied. Each study was also evaluated as to how and where it might fit in an adverse outcome pathway (OECD 2011; 2013) based on the outcome (e.g., molecular initiating event, cellular response, organ response, or organism response). Epidemiologic studies were similarly evaluated first with respect to data quality and study design, and second as to whether the study provided evidence for or against synergistic effects. Relevant genes identified from the comparative toxicogenomics database were explored to determine potential targets and commonalities across the four metals and subsets of metals. Where possible, we compare *in vivo* doses or body burdens from the toxicological and epidemiologic studies to NHANES or other biomonitoring data from the peer-reviewed literature.

3.0 RESULTS

3.1 Toxicological Studies

Table S1 provides a summary of toxicological studies related to developmental or neurotoxic endpoints for mixtures of metals. The table also includes studies that focused on metal disposition in the body and how combined exposures might influence biologically-relevant doses at target tissues, including concentrations in the brain.

Hypothesized modes of action for individual constituent toxicity provide a baseline for understanding potential mechanisms associated with combined exposures. All of these metals interfere with neuronal signaling mechanisms in various ways. For example, lead interferes with calcium homeostastic and calcium-regulated cellular transport and communication (Schwartzer et al. 2013; Hu et al. 2007). Lead, as a divalent cation, readily bonds to oxygen and sulfur and competes with calcium for common binding sites. Exposure to lead activates protein kinase C (PKC) and studies have shown increases in intracellular calcium levels leading to production of free radicals and reactive oxygen species (ROS) as excessive calcium enters mitochondria. Lead also interferes with N-methyl-d-aspartate (NMDA) receptor function in the hippocampus and long term potentiation (Alkondonet et al. 1990), a mechanism of synaptic

plasticity. Rodent performance on a variety of behavioral tests (e.g., Morris water maze test and others) is known to be dependent on these receptors. Glutamatergic neurotransmission and GAGAergic neurotransmission are impaired following exposure to lead (Schwartzer et al. 2013).

The choroid plexus, the blood-cerebrospinal fluid (CSF) barrier which manufactures and secretes proteins for the extracellular compartment of the central nervous system, is another target for lead (Zheng 2001). The choroid plexus separates systemic blood circulation for most tissues and organs from the brain. Arsenic and cadmium accumulate in the choroid plexus and can cause structural damage, allowing metal transport into the brain and facilitating the transport of other constituents (Zheng 2001). Pb and Mn do not directly alter permeability, but act on critical regulatory functions of the choroid plexus. For example, chronic exposure to lead reduces levels of transthyretin (TTR or prealbumin) protein levels responsible for transporting thyroid hormones and retinol (Zheng 2001).

As is a known mitochondrial phosphorylation uncoupler. As(III) molecules have a strong affinity to sulfydryl groups of tissue proteins, while As(V) mimics oxyanion phosphate. Both inhibit mitochondrial energy production. As(III) increases oxidative stress in the mouse brain, alters neural networking, and leads to an increase in reactive oxygen intermediates in brain explants and neuronal cell cultures (Wright et al. 2006). Exposure to Mn is also associated with oxidative damage to neurons and effects on essential neuronal metabolic enzymes.

3.1.1 Neurotransmitters and Metabolites

The effects of Pb and As on levels of neurotransmitters and their metabolites were studied in mice (Mejía et al. 1997). Lead acetate (74 mg Pb/kg/day) and sodium arsenite (8.0 mg As/kg/day) were administered by gavage separately and together to adult male mice for 14 days. Six areas of the brain (hypothalamus, medulla, pons, midbrain, striatum, hippocampus, and cortex) were examined. Arsenic alone generally increased the concentration of dopamine and serotonin and their metabolites and decreased norepinephrine in the brain areas. The only significant effect of lead alone was an increase in 3,4-dihydroxyphenyl-acetic acid, a metabolite of dopamine, in the hypothalamus. The mixture produced effects similar to those of arsenic alone except for an increase in serotonin in the cortex and midbrain and a decrease in norepinephrine in hippocampus that were significant, and greater than the slight change in the same direction seen with either metal alone. The selective increase of serotonin due to exposure to a mixture of Pb and As was not observed in response to either metal alone. Serotonin and

acetylcholine are primarily responsible for modulating cortical activity and form the basis of arousal and cognitive function.

Rodriguez et al. (1998) compared *in vivo* dopamine release from adult rats subchronically exposed to mining waste and found significantly decreased basal levels of dihydroxyphenylacetic acid but comparable dopamine release rates. The mining waste group, however, were not able to sustain increased dopamine release in response to depolarization. Groups of rats exposed simultaneously to Mn through drinking water (3 mg/ml) and Pb intraperitoneally (lead acetate at 4.0, 8.0 and 12.0 mg/kg) daily for a period of 14 days (Chandra et al. 1981) found significant decreases (greater than additive) in serotonin as compared to exposure to either Mn or Pb individually. The decrease was only observed at the highest concentration of Pb. This study demonstrated a greater than additive effect, which may be protective of impacts given that reduced serotonergic function may facilitate learning (Meneses 1998). By comparison, Desole et al. 1995 found that exposure to Mn alone decreased seratonin levels in rat striatum but not significantly from controls, while in older rats, serotonin levels were increased at lower Mn doses (30 mg/kg-d) and unaffected at higher Mn dose levels (200 mg/kg-d). The increase in serotonin at lower Mn doses (with or without concurrent Pb exposure) is consistent with that observed for Mn in Chandra et al. (1981), suggesting an interaction between Pb and Mn related to serotonin production.

An *in vitro* study in rat brain synaptosomes (Chandra et al. 1984) studied the effect of individual and combined exposures to Cd^{2+}, Pb^{2+} and Mn^{2+} and found that lower concentrations of Pb^{2+} alone increased while higher concentrations inhibited synaptosomal uptake of labeled dopamine (3H-DA) and labeled noradrenaline (3H-NA). Lower concentrations of Cd^{2+} increased the uptake of 3H-DA while at concentrations of 100 µM, uptake was inhibited. Mn^{2+} inhibited the uptake of labelled amines, and the interaction of Mn^{2+} with Pb^{2+} or Cd^{2+} produced greater-than-additive inhibition on the uptake of 3H-DA and 3H-NA.

A study of intraperitoneal exposure over 30 days to Mn (4 mg/kg), Cd (0.5 mg/kg), Zn (1 mg/kg) and Hg (0.5 mg/kg) in Albino rats found significant increases in dopamine and norepinephrine in the brain, but no change in serotonin levels as compared to levels from exposure to individual constituents (Shukla and Chandra 1982). Simultaneous exposure to Mn and Pb produced significant hypoactivity in rats exposed subchronically (Chandra et al. 1981) which was related to a decrease in the norepinephrine contents in the brain. Antonio et al. (2002) exposed pregnant rats to Pb in drinking water (300 mg/l) and

Cd (10 mg/l) throughout pregnancy and studied the pups. At weaning, Pb and Cd produced an increase in neurotransmitters in the cerebellum, although contents in the striatum remained unaltered. There was a direct correlation with decreased performance on the open-field motor activity test.

3.1.2 Cellular Signaling

An *in vitro* study of individual and combined exposures to Cd^{2+}, Pb^{2+} and Mn^{2+} in rat brain synaptic plasma membranes found that exposure to Cd^{2+}, Pb^{2+} and Mn^{2+} individually inhibited synaptosomal Na^{+}/K^{+}-ATPase activity (Carfagna et al. 1996). This study found that simultaneous exposure to the combinations of $Cd^{2+}$and Mn^{2+} or Pb^{2+} and Mn^{2+} inhibited Na^{+}/K^{+}-ATPase synergistically, while $Cd^{2+}$and Pb^{2+} caused additive inhibition. Simultaneous exposure to Cd^{2+} and Pb^{2+} antagonistically inhibited Mg^{2+}-ATPase activity while Cd^{2+} and Mn^{2+} or Pb^{2+} and Mn^{2+} additively inhibited Mg^{2+}-ATPase activity at low Mn^{2+} concentrations, and inhibited antagonistically at higher Mn^{2+} concentrations. Chandra et al. (1984) also studied the *in vitro* effect of individual and combined exposures to Cd^{2+}, Pb^{2+} and Mn^{2+} in rat brain synaptosomes and found the inhibition of Na^{+}/K^{+}-ATPase activity to be concentration-dependent for Cd^{2+} and Pb^{2+} but observed no effect of Mn^{2+}. Interaction of Cd^{2+} with either Mn^{2+} or Pb^{2+} showed the greatest inhibition of synaptosomal transport.

Hussain et al. (1987) studied the interaction of Pb^{2+} and Mn^{2+} on Na^{+}/K^{+}-ATPase activity in rat striatal synaptosomes *in vitro* with and without lipoperoxidation and found an additive decrease in Na^{+}/K^{+}-ATPase activity in untreated cells and a greater than additive decrease in treated cells. In a series of *in vitro* experiments with rat synaptosomes from the forebrain and striatum and individual concentrations of Cd^{2+}, Mn^{2+}, and Al^{2+}, Lai et al. (1980; 1981) found that each of these inhibited Na^{+}/K^{+}-ATPase activity, but did not test for any combined effects. Na^{+}/K^{+}-ATPase activity was significantly inhibited in all brain regions in rat pups maternally exposed through drinking water to Pb (300 mg/l) and Cd (10 mg/l) throughout gestation and lactation, with a concomitant decrease in motor activity tests (Antonio et al. 2002).

In a study of rat pups exposed throughout gestation and lactation to a combination of Pb, As, and Cd, Rai et al. (2010) observed a greater than additive reduction in glial fibrillary acidic protection (GFAP) expression during brain development and concomitant reductions in learning and memory performance. GFAP-generating astrocytes were greater than additively reduced, and an exploration of MAPK signaling pathways revealed a greater than additive downregulation in viability and an increase in apoptosis of

astrocytes, induced by proximal activation of extra cellular signal-regulated kinase (ERK) signaling and downstream activation of the Jun N-terminal kinase (JNK) pathway. This led to an increase in Ca^{2+} release and reactive oxygen species generation, resulting in apoptosis in the astrocytes.

3.1.3 Other Responses

Heme biosynthesis represents a shared target for the effects of all four metals. Andrade et al. (2013) treated five groups of rats for 8 days with individual concentrations of Pb (mg/kg), As (60 mg/L) and Mn (10 mg/kg) as well as the combination. Co-treated rats showed a statistically significant correlation between increased Pb, As, Mn and delta-aminolevulinic acid (delta-ALA) levels in the brain and decreased motor activity. Urinary delta-ALA concentrations were higher in the mixture-treated group than the sum of the delta-ALA levels in each single-treated group, and also found a significant correlation with brain delta-ALA levels. Delta-ALA is the first precursor in heme synthesis in a reaction catalyzed by deltaaminolevulinic acid synthetase (ALAS). Decreased heme formation stimulates ALAS via a negative feedback loop, resulting in increased delta-ALA levels in both circulating blood and urine, as was observed in this study.

In a 10-week dietary study at 10 mg Pb/kg/day and 2.5 mg As/kg/day in young adult male rats, hemoglobin was slightly decreased and hematocrit was significantly decreased by As alone, but not by Pb alone or the Pb-As mixture, indicating a less-than-additive effect for the mixture (Mahaffey and Fowler 1977; Mahaffey et al. 1981).

3.1.4 Absorption, Metabolism, Distribution, and Elimination

Rodriguez et al. 1998 exposed 60 adult male Wistar rats to mining waste (containing a mixture of, in descending order, As, Mn, An, Cu, Pb, Ni, Sb, Ba, Cr, and Cd) or plain arsenite. Experimental and control animals were also given feed pellets, which also contained detectable concentrations of As, Mn, and Pb. Although Mn concentrations in the mining waste and food pellets was approximately 100 times lower than that of As, measured Mn concentrations in the brain were on the same order of magnitude as those of arsenic. Chandra et al. 1981 found increases in lead concentrations in the brain following simultaneous exposure to Mn via drinking water (3 mg/ml) and lead via food (5.0, 8.0, 12.0 mg/kg) as compared to lead alone at the same concentrations.

In animal studies involving combined exposures to both Pb and Mn, brain Pb levels were significantly higher in mixture-exposed animals in adults (Chandra et al. 1981) and developmentally exposed (Chandra et al. 1983) at relatively higher exposure levels. This was observed in maternally-exposed pups at comparatively lower exposure levels (Betharia and Maher 2012) at post-natal days 2 and 25, but at post-natal day 60, there were no differences across individually- and mixture-exposed groups. However, Betharia and Maher (2012) did observe a pharmacokinetic interaction between Pb and Mn for excretion through milk, which has not been observed elsewhere.

In a 10-week dietary study at 10 mg Pb/kg/day and 2.5 mg As/kg/day in young adult male rats, neither metal affected tissue distribution of the other (kidney, liver, brain, and bone concentrations), relative to distribution following dietary exposure to the single metal at the same dose level as in the mixture (Mahaffey et al. 1981). Pb was not detected in the liver or brain. In addition, each of these two metals increased urinary coproporphyrin excretion, and the effect of the mixture was additive. Uroporphyrin excretion was increased by arsenic and not affected by lead; results from the mixture were the same as for arsenic alone (no apparent interaction) (Fowler and Mahaffey 1978; Mahaffey et al. 1981).

The effects of Pb and As on each other's distribution to the brain were studied in mice (Mejía et al. 1997). Lead acetate (74 mg Pb/kg/day) and sodium arsenite (8.0 mg As/kg/day) were administered by gavage separately and together to adult male mice for 14 days. Six areas of the brain (hypothalamus, medulla, pons, midbrain, striatum, hippocampus, and cortex) were examined. Concentrations of As in the brain areas were decreased by coexposure to Pb (significantly in four of the areas), and those of Pb were increased by coexposure to arsenic (significantly in three of the areas), relative to concentrations resulting from exposure to individual metals.

An intraperitoneal administration of Mn (4 mg/kg) in male Albino rats over 30 days enhanced the uptake of Cd (0.5 mg/kg) in the brain (Lal et al. 1980).

Gu et al. (2008) observed a greater than additive effect on DMT1 protein synthesis, enhancing transport of ions in the developing rat brain resulting from exposure to both Cd (2 mg/kg-d) and Pb (20 mg/kg-d) via intragastrical perfusion of Sprague-Dawley rats once a day for six weeks.

Summary of Toxicological Studies

The combined evidence suggests that the combination of Mn with other metals increases the concentrations of the other metals, for example, coadministration of Pb and Mn increases brain Pb levels by a factor of three as compared to lead alone (Chandra et al. 1981) and increased brain Pb in five of seven brain regions (Shukla and Chandra 1987). Mining waste containing a mixture of Pb, Mn and As given to rats resulted in elevated levels of brain As and Mn (Rodriguez et al. 1998). Andrade et al. (2013) found increased levels of Pb, Mn, and As in rat brains following coadministration as compared to uptake by individual metals, and found a correlation with Delta ALA-U levels in urine. The combined administration of Mn and Cd increased the brain contents of Cd as compared to individual uptake in a study of rates exposed intraperitoneally (Lal et al. 1980).

Mining waste containing a mixture of Pb, Mn and As decreased dopaminergic transmitter following depolarization (Rodriguez et al. 1998). Similarly, Mejía et al. (1997) demonstrated greater than additive changes in monoaminergic neurotransmitter levels in rat brains following administration of Pb and As. Leret et al. (2003) found similar results in a study of pregnant rats exposed to Pb and Cd, although the design of this study was such that it was not possible to determine whether the interaction was greater than additive or not. Chandra et al. (1984) in an *in vitro* study of rat synaptosomes found an interaction between Mn and Pb or Cd and dopamine and noradrenaline uptake.

In terms of potential pathways, greater than additive effects have been observed for some combinations of metals. Antonio et al. (2002) observed significant inhibition of Na^+/K^+-ATPase activity *in vivo* following coadministration of Pb and Cd, while Carfagna et al. (1996) noted greater than additive action of Pb or Cd in the presence of Mn, and Hussain et al. (1997) noted the same for Pb and Mn in rat synaptosomes *in vitro*. However, Chandra et al. (1984) in an *in vitro* study of rat synaptosomes noted only additive interactive effect of Pb, Mn and Cd with no effect of Mn, and Lai et al. (1980) noted no effects at all of Cd, Mn, and Al exposures.

Rai et al. (2010) observed a greater than additive downregulation in viability and increased apoptosis of astrocytes, induced by ERK/JNK signaling, leading to an increase in Ca^{2+} and ROS generation.

4.0 EPIDEMIOLOGIC STUDIES

The literature search to identify epidemiologic studies that examined associations of neurodevelopment with exposure to at least one of the metals of interest (Pb, Cd, Mn, As) and/or to other toxicants identified 22 peer-reviewed studies, summarized in Table S2. Of these 22 studies, seven measured multiple metals, but did not examine interactive effects of metals on neurodevelopmental outcomes. Fourteen of the 22 studies examined interactions between the metals of interest listed above, and one study evaluated interactions between a metal of interest and another toxicant. The majority of studies limited the interaction analyses to binary combinations of metals. Furthermore, there is little consistency in the exposure measures and the statistical approach used to examine interactions. Despite the relative lack of consistency between studies, at least one trend seems to be present in the existing literature: the toxicity of lead, a well-documented neurotoxicant, appears to increase in the presence of high levels of other metals (e.g., Mn, As, Cd). We summarize results from these studies by neurodevelopmental outcome, or by general domain affected.

Four studies examined autism spectrum disorder in relation to exposure to multiple metals (Adams et al. 2009, Geier et al. 2012, Roberts et al. 2013, Obrenovich et al. 2011). Adams et al. examined urinary Pb, As, and Cd, among other metals, as predictors of autism severity in a small, cross-sectional study of 3-8 year old children. Associations of each individual metal with autism were examined, without consideration of confounding by other metals. While urinary levels of Pb (and Sb) were observed to be most strongly associated with autism severity, authors report high correlations between metals and thus suggest interpreting results as evidence for a general role of toxic metals in relation to autism severity rather than evidence for any particular metal. Geier et al. 2012 also examined autism spectrum disorder severity in a cross-sectional study of 18 children. Hg measured in hair was the only metal shown to correlate with increased ASD severity. Confounding by other metals was also not considered. Roberts et al. 2013 evaluated metals (and other toxicants) in air pollution in relation to ASD among offspring of the Nurses' Health Study, a longstanding longitudinal prospective cohort study. Authors noted significant associations of Pb, Mn, and an overall measure of metals with ASD. Among the 3 studies of ASD evaluated here, none explicitly evaluated interactions between metals, though Roberts et al. examined an overall measure of metals, which may be considered akin to a model of additive interaction.

All of the aforementioned studies have suggested that oxidative stress, which has been implicated in autism, may play a substantial role in the mechanistic pathway between metals exposure and the

disorder. Adams et al. suggest that, because oxidative stress and thiol metabolic disturbances have both been described in the autism population, it is likely that these play a role in both relative burden and susceptibility to heavy metals. Heavy metal exposure also generates oxidative stress and thiol depletion. Further, prior depletion of thiols and increased oxidative stress makes it more likely the individual will accumulate metals. Geier et al. (2012) similarly hypothesized that the impact of metals may be stronger among patients with severe ASD compared to those with mild ASD, and that excretion pathways may be different in severe vs mild ASD cases. Furthermore, Obrenovich et al. (2011) reported that, compared to age-matched healthy controls (n=39), children with ASD (n=26) demonstrated abnormal markers of thiol metabolism, higher levels of hair As, and lower levels of hair Hg, Cu, and Fe. Altered thiol metabolism is a mechanism for oxidative stress, whereby diminished antioxidant capacity is mediated by thiol-rich enzymes (Aliev et al. 2008; 2009). Taken together, these studies suggest that one potential pathway between exposure to multiple metals and autism spectrum disorder may be through altered thiol metabolism and increased oxidative stress. In a cross-sectional study of 10,098 adults, Lee et al. (2006) found an association between blood Pb and urinary Cd and oxidative stress markers based on NHANES data.

We reviewed 14 studies evaluating exposure to multiple metals and associations with cognitive and motor development. Two of the 14 studies (Parajuli et al. 2013; Thatcher et al. 1982) adjusted for metal co-exposures, but did not evaluate interactions between metals; one of the 14 studies (Boucher et al. 2012) evaluated interactions but for Pb with other toxicants that are outside the scope of this review. Eleven of the 14 studies evaluated interactive effects of our metals of interest (Pb, Cd, As, Mn). In relation to mental and/or motor development, four of the 11 studies examined interactions between Mn and Pb exposure (Kim et al. 2009; Claus Henn et al. 2012; Lin et al. 2013; Lucchini et al. 2012); three examined interactions between Mn and As (Khan et al. 2011; Wasserman et al. 2011; Wright et al. 2006); one evaluated Pb-Cd interactions (Kim et al. 2013); and three considered Pb-As interactive effects (Calderon et al. 2001; Rosado et al. 2007; Moon et al. 1985).

In 2009, Kim et al. reported findings from a cross-sectional study of 261 Korean children, aged 8-11 years. Authors reported significant associations between blood Pb levels and full-scale as well as verbal IQ, though only among kids with high blood Mn levels, suggesting significant effect modification of Pb by Mn. Claus Henn et al. (2012) reported similar findings in a prospective cohort of 450 Mexican children between one and three years of age. In this study, blood Mn and Pb levels were associated with repeated measures of neurodevelopment, assessed with the Bayley Scales of Infant Development II

(BSID-II). The BSID evaluates mental development (sensory/perceptual acuities, discriminations, acquisition of object constancy, memory, learning and problem-solving, vocalisation, early verbal communication and abstract thinking, habituation, mental mapping, complex language and mathematical concept formation) and motor development (degree of body control, coordination of large muscles, fine manipulation skills, dynamic movement, postural imitation and stereognosis). Increased Pb toxicity was observed among children with high blood Mn levels, compared to children with lower blood Mn levels. A third study conducted by Lin et al. (2013) also reported interactions between Mn and Pb, whereby significantly lower scores on the Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) were observed among 230 two-year-old children whose cord blood Mn and Pb levels were both high (>75th percentile), as compared to children with low cord blood Mn and Pb levels. This study additionally adjusted for cord blood As and Hg levels as confounders. In contrast to the three aforementioned studies, Lucchini et al. (2012) observed no interactions between blood Pb and either blood, hair, air, or soil Mn in relation to cognitive function. Authors reported significant adverse effects of Pb on IQ, measured using the Wechsler Intelligence Scale for Children (WISC), among 299 adolescents (11-14 years old) residing near a ferro-manganese plant in Italy, but no Mn-Pb interactions were observed.

It has been suggested that Mn and Pb cause synergistic effects on neurodevelopment due to their interactions with similar proteins. Pb disrupts zinc in regulation of NMDA receptor activation, and inhibits Ca^{2+} dependent acetylcholine and dopamine release. At high levels, Mn affects dopaminergic and cholinergic neurotransmitters and synaptic modulation, inhibits various protein transports and enzymes, and can increase release of nitric oxide, which is involved in cellular signal transduction. At insufficiently low levels, Mn may also have adverse neurodevelopmental effects. Although evidence points to an inverted U-shaped relationship between Mn and neurological function (Claus Henn et al. 2010), Claus Henn et al. (2012) found no change in Pb toxicity among children with low Mn levels, suggesting that mechanisms by which Mn deficiency causes adverse neurological effects may not overlap with those for Pb.

In a small pilot study of 32 children ages 11-13 years, Wright et al. (2006) reported a significant interaction between hair As and Mn levels on full-scale and verbal IQ, as well as on verbal memory. In 2011, two other cross-sectional studies examined Mn-As interactive effects, both among children in Bangladesh, but observed no significant interactions. Khan et al. (2011) investigated associations of Mn and As concentrations measured in tube well water with classroom behavior, specifically internalizing

and externalizing behavior. After adjusting for co-exposures, main effects of Mn on internalizing and externalizing behaviors were reported, but no statistical interactions for Mn-As were observed. Wasserman et al. (2011) similarly examined Mn-As interactive effects on intelligence among 299 Bengali children, but found no evidence of interaction between these metals measured in blood.

Two studies of which we are aware examined interactive effects of Pb and Cd on neurodevelopment. Marlowe et al. (1985b) investigated associations between hair metals and metal combinations with children's visual-motor performance, assessed with the Bender Visual-Motor Gestalt Test, in 69 elementary school age children. Various binary combinations of metals were evaluated using partial regression coefficients. Using hair as the exposure index, the combination of Pb and Cd together, among other binary combinations, were found to account for a significant proportion of variance in classroom teachers' ratings. Kim et al. (2013) dichotomized blood Cd exposure at the median, and examined effects of blood Pb levels at low vs high Cd levels. Neurodevelopment was assessed at 6 months of age using Bayley Scales of Infant Development. Authors reported a potential antagonistic interaction between Pb and Cd in maternal blood during early pregnancy for mental development scores. During late pregnancy, however, authors observed synergistic effects, whereby adverse Pb effects on mental and psychomotor development scores were only significant at high Cd levels. In terms of possible mechanism, Pb and Cd have been shown to cause oxidative stress in astrocytes (Lee et al. 2006; Yang et al. 2008), and to interfere with calcium signaling (Rai et al. 2010) and neuroendocrine controls such as thyroid hormone (Ishitobi et al. 2007; Lamb et al. 2008). The antagonistic interaction between Pb and Cd reported here may be due to a potential protective effect of Cd at low levels, and/or an interference of Cd with Pb uptake, which has been demonstrated in pregnant mice (Smith et al. 2012). Cd has been shown to stimulate ovarian progesterone biosynthesis at low levels, but to inhibit the same hormone at high levels (Henson and Chedrese 2004). Cd has also been found to stimulate DNA synthesis and cell proliferation at low levels but to increase apoptosis and chromosomal aberrations at high levels. While these results need to be confirmed in other studies, the shift in direction of the Pb-Cd interaction observed here suggests that mechanisms for interaction between these metals may depend on stage of pregnancy and/or dose of Cd.

Results from studies examining Pb-As interactive effects on neurodevelopment are mixed. In a correlation analysis, Moon et al. (1985) and Marlowe et al. (1985b) reported interactions between Pb and As, measured in hair, with cognitive function (Wide Range Achievement Test [WRAT] reading and spelling tests, and Bender Visual-Motor Gestalt Test) in 69 elementary school age children. In these

studies, decreased reading and spelling achievement were associated with higher hair Pb and As levels, and the combination of As and Pb together was found to account for a significant proportion of the variance in classroom teachers' ratings on the Bender Visual-Motor Gestalt Test. In contrast, Calderon et al.(2001) found no evidence of interactions between urinary As and blood Pb in a cross-sectional study on associations between Pb, As, and the Wechsler Intelligence Scale for Children (assessing full, verbal, performance IQ; long-term memory; linguistic abstraction; attention span; and visuospatial organization). Details about how interaction was assessed, however, were not provided. Rosado et al. (2007) reportedly considered As-Pb interaction in regression models, but did not report any results, leading reader to assume that no significant interaction was observed.

Finally, we reviewed five epidemiologic studies that examined behavioral outcomes in relation to multiple metals exposure. Four of these studies evaluated binary metal interactions (Lucchini et al. 2012; Wright et al. 2006; Marlowe et al. 1985a; Khan et al. 2011); one study did not evaluate interactions (Haynes et al. 2011). Lucchini et al. (2012) observed no interactions between blood Pb and either blood, hair, air, or soil Mn in relation to behavior, assessed using the Conners-Wells' Adolescent Self-Report Scale Long Form (CASS:L), in a cross-sectional study of 299 Italian adolescents. Marlowe et al. (1985a) investigated associations between hair metals, individually and in binary combinations, with children's classroom behavior assessed with the Walker Problem Behavior Identification Checklist (for nonadaptive classroom behavior). Among 80 elementary school age children, a significant increase in explained variance was observed due to Pb-As and Pb-Cd interactions. No significant Mn-As interactive effects on behavioral outcomes were observed in either of the two studies examining these associations (Wright et al. 2006; Khan et al. 2011). Khan et al (2011) evaluated associations of Mn and As in tube well water with classroom behavior, specifically internalizing and externalizing behavior. No statistical interactions for Mn-As were observed.

4.1 Exposure Levels from the Epidemiologic Studies as Compared to Biomonitoring Data

The National Health and Nutrition Examination Survey (NHANES) database provides data on urinary and blood levels of As, Pb, and Cd for the 2009-2010 sampling timeframe. No NHANES data are available for Mn. In an exploratory analysis, we compare individual levels of As, Pb, and Cd from epidemiologic studies with NHANES data to determine the potential overlap with concentrations associated with effects and observed values in the general population.

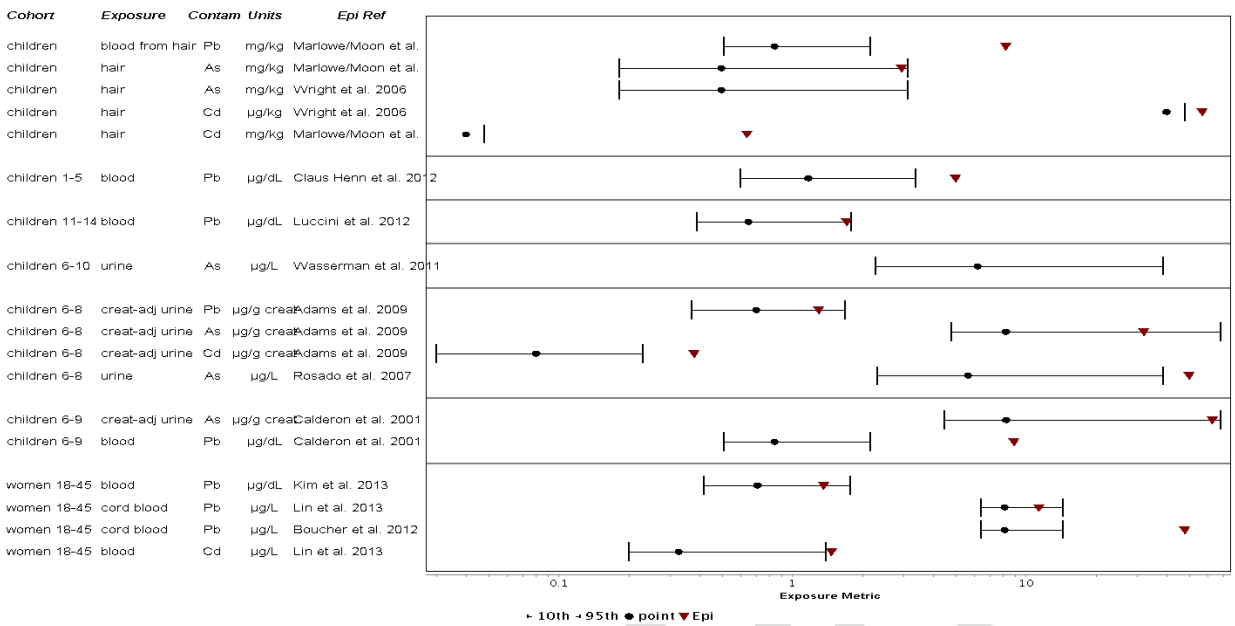

Figure 3: Comparison of NHANES Biomonitoring Data to Exposure Concentrations in Selected Epidemiologic Studies

The epidemiologic studies are based different exposure metrics, including concentrations of metals in hair, urine (both creatinine and non-creatinine adjusted), maternal or child blood, and cord blood. NHANES data are only for urine and blood. In order to be able to use the hair data, we use regression relationships from the literature to relate blood or urine levels to hair levels (Baker et al. 1979; Esteban et al. 1999; Shraim et al. 2003; Blaurock-Busch et al. 2011) and maternal blood levels to cord blood (Zarembski et al. 1983; Ong et al. 1985). Data for manganese from a study in Maine based on blood levels in one to five year olds (Rice et al. 2010) shows that the 95th percentile level observed in that population is below levels from the epidemiologic studies.

Figure 3 shows these results of the comparisons between biomonitoring data and exposure levels from the epidemiologic studies that evaluated mixtures. This initial screening comparison shows that, on an individual chemical basis, exposures from the epidemiologic studies are either at or exceed the 95th percentile of population exposures.

5.0 COMPARATIVE TOXICOGENOMICS DATABASE

Table 1 summarizes the results of the literature search to identify curated genes implicated across As, Pb, Mn, and Cd exposures associated with several learning disorders, as well as iron metabolism. These genetic targets of metal action provide insight into potential pathways by which *in vivo* effects are

observed, recognizing that it is highly unlikely that mixtures of metals act on the same pathways in the same ways to result in greater than additive effects. Nonetheless, common cellular targets provide evidence that combined exposures could result in synergistic activation or deactivation of pathways relevant to expected neurodevelopmental physiological functions. Genes shown in red are common targets across all four metals; blue are shared by three as shown in Table 1, below. A minus means deactivation of the gene or protein regulated by the gene or a decrease in mRNA; plus means activation of the gene or protein or a decrease in mRNA.

Table 1: Top Interacting Genes from the Comparative Toxicogenomics Database (www.ctdbase.org)

Genes	Pathway	As	Pb	Mn	Cd	Top Ten Interacting ?
ACHE	Acetylcholinesterase hydrolyzes the neurotransmitter, acetylcholine at neuromuscular junctions and brain cholinergic synapses, terminating signal transmission. It is also found on the red blood cell membranes, where it constitutes the Yt blood group antigen.	-	-	no data	-	
ANXA2	Annexin family. Members of this calcium-dependent phospholipid-binding protein family play a role in the regulation of cellular growth and in signal transduction pathways.	var	-	+	+	
BDNF	BDNF signaling pathway; neurotrophin essential for growth, differentiation, plasticity, and survival of neurons. BDNF is also required for processes such as energy metabolism, behavior, mental health, learning, memory, stress, pain and apoptosis. Signal transduction, MAPK pathway.	-	-	+	-	
CASP3	Encodes a protein in the cysteine-aspartic acid protease (caspase) family. BDNF signaling pathway; caspase cascade in apoptosis. Associated with neuronal death in Alzheimer's, Parkinsons. MAPK signaling pathway. Signal transduction in autism.	+	+	+	+	Mn, Cd
CAT	Encodes catalase, a key antioxidant enzyme in the bodies defense against oxidative stress. Catalase is a heme enzyme that is present in the peroxisome of nearly all aerobic cells.	-	-	-	-	As, Cd
CCL2	One of several cytokine genes. Chemokines are a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes.	+	+	+	+	As (inc susc)

FOS	Encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. In some cases, expression of the FOS gene has also been associated with apoptotic cell death.	+	+	+	+	
GSR	Encodes a member of the class-I pyridine nucleotide-disulfide oxidoreductase family. This enzyme is a homodimeric flavoprotein. It is a central enzyme of cellular antioxidant defense, and reduces oxidized glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant.	-	var	var	var	
HMOX1	Heme oxygenase, an essential enzyme in heme catabolism, cleaves heme to form biliverdin, which is subsequently converted to bilirubin by biliverdin reductase, and carbon monoxide, a putative neurotransmitter. Heme oxygenase activity is induced by its substrate heme and by various nonheme substances.	+	+	+	+	Pb, As, Cd; Pb, As poly inc susc
HSPA5	Encodes a member of the heat shock protein 70 (HSP70) family. Localized in the lumen of the endoplasmic reticulum (ER); involved in the folding and assembly of proteins in the ER.	var	+	+	+	Pb
HSPA8	Encodes a heat-shock cognate protein. Functions as a chaperone, and binds to nascent polypeptides to facilitate correct folding. Functions as an ATPase in the disassembly of clathrin-coated vesicles during transport of membrane components through the cell.	+	+	+	+	
IL1B	Enclodes interleukin 1 cytokine family. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). Important mediator of the inflammatory response, including cell proliferation, differentiation, and apoptosis.	+	+	+	+	
IL8	Encodes a member of the CXC chemokine family, major mediator of inflammatory response.	+	+	+	+	
MAPK1	MAP kinases, also known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals. Involved in proliferation, differentiation, transcription regulation and development. Activation requires phosphorylation by upstream kinases. Once activated, kinase translocates to the nucleus of the stimulated cells, where it phosphorylates nuclear targets.	+	var	+	+	Mn, As, Cd

MAPK3	MAP kinases, also known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals. Involved in proliferation, differentiation, transcription regulation and development. Activation requires phosphorylation by upstream kinases. Once activated, kinase translocates to the nucleus of the stimulated cells, where it phosphorylates nuclear targets.	+	+	+	+	Mn, As, Cd
MMP2	Encodes a protein in the matrix metalloproteinase (MMP) family; involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling.	+	+	-	no data	
NFE2L2	Encodes a transcription factor which is a member of a small family of basic leucine zipper (bZIP) proteins. The encoded transcription factor regulates genes which contain antioxidant response elements (ARE) in their promoters; many of these genes encode proteins involved in response to injury and inflammation which includes the production of free radicals.	+	+	+	+	As (inc susc), Cd (dec susc)
NOS2	Nitric oxide is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities. Involved in calcium signaling.	-	+	+	+	
NOS3	Nitric oxide is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities. Involved in calcium signaling.	-	+	+	+	polymorph in As leads to inc susc
NQO1	Member of the NAD(P)H dehydrogenase (quinone) family and encodes a cytoplasmic 2-electron reductase. This FAD-binding protein forms homodimers and reduces quinones to hydroquinones. Associated with Alzheimer's disease.	+	+	+	+	
OGG1	Encodes the enzyme responsible for the excision of 8-oxoguanine, a mutagenic base byproduct from exposure to reactive oxygen. The action of this enzyme includes lyase activity for chain cleavage.	var	-	-	-	As, Cd
PPARG	Encodes peroxisome proliferator-activated receptor PPAR-gamma and is a regulator of adipocyte differentiation. Additionally, PPAR-gamma has been implicated in the pathology of numerous diseases including obesity, diabetes, atherosclerosis and cancer.	-	+	+	-	

PTGS2	Prostaglandin-endoperoxide synthase (PTGS), cyclooxygenase, is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. Inducible isozyme regulated by specific stimulatory events; responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis.	**var**	**+**	**+**	**+**	
SLC11A2	Encodes a member of the solute carrier family 11 protein family. Transports divalent metals and is involved in iron absorption. Mutations in this gene are associated with hypochromic microcytic anemia with iron overload.	inc uptake	inc uptake	inc uptake	inc uptake	Mn
TH	Encodes protein involved in the conversion of tyrosine to dopamine. Rate-limiting enzyme in the synthesis of catecholamines, hence plays a key role in the physiology of adrenergic neurons.	**-**	**-**	**-**	**+**	

6.0 PATHWAY FROM MOLECULAR INITIATING EVENTS TO HEALTH OUTCOME

Following guidance developed by the Organisation for Economic Co-operation and Development (OECD 2011; 2013), several adverse outcome pathways are proposed to identify potential pathways of causal linkages between several molecular initiating events and final adverse effects at a biological level of organization that is relevant to a regulatory decision. The proposed AOPs are based on an integrated and systematic assessment of the toxicological and epidemiologic data on combined exposures of two or more of the metals of concern. Figure 4 provides a general schematic of potential key events.

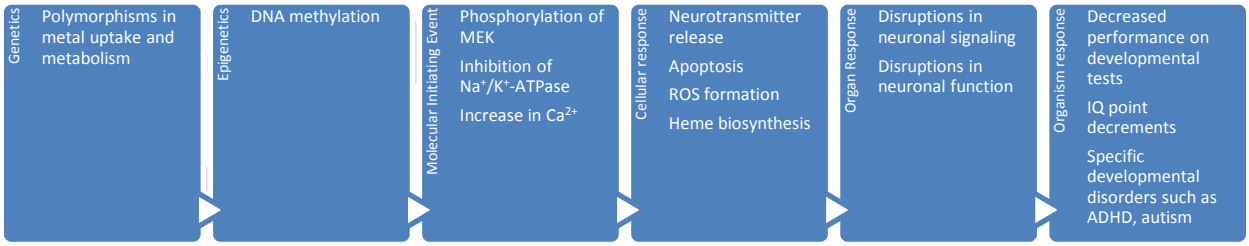

Figure 4: Linkages from Molecular Initiating Events to Adverse Outcomes

Table 2 provides a summary of several proposed molecular initiating events leading to cellular and organ responses that ultimately manifest in observations of decreased performance on a battery of behavioral and developmental tests in humans and animals correlated with concentrations of two or more metals.

The emphasis is on mechanisms by which exposure to two or more metals might lead to effects greater than would be predicted by assuming additivity or independence of individual metals. Based on an evaluation of the evidence, there are several opportunities for exposure to metals to influence synaptic connections between nerve cells as they are being established during brain development. Most of these ultimately relate to calcium signaling which directs structural as well as functional adaptation in individual neurons to establish synaptic selectivity in the developing brain (Lohmann 2009; Michaelson and Lohmann 2010). Glial cells, and in particular astrocytes, are also documented targets for mixtures of metals, and while astrocytes have long been considered to support neuronal signaling, there is increasing evidence that human astrocytes detect synaptic activity and engage in reciprocal signaling with neurons, again based on intracellular Ca^{2+} variations (Navarette et al. 2013). Astrocyte contact is necessary for the synaptic maturation of developing neurons (Barker and Ullian 2008), and a recent study shows that neuroglial signaling involving astrocytes differs significantly in the adult versus developing brain (Sun et al. 2013), highlighting the potential vulnerability of perinatal exposures .

Molecular Initiating Event: Rai et al. (2010) treated astrocytes with a mixture of As, Cd and Pb, and observed synergistic downregulation in viability and increased astrocytic apoptosis induced by proximal activation of extra cellular signal-regulated kinase (ERK) signaling and downstream activation of the Jun N-terminal kinase (JNK) pathway. Therefore, phosphorylation of MEK1/2 represents an initiating molecular event leading to activation of JNK pathways. Data from the Comparative Toxicogenomics Database shows that all four metals independently are documented to increase the expression of MAPK3, and As, Mn and Cd increase the expression of MAPK1 with a variable response from Pb. In addition, both MAPK1 and MAPK3 are in the top ten interacting genes for As, Mn, and Cd. All four metals (independently) also increase the expression of CASP3, which is associated with the BDNF and MAPK signaling pathways, the caspase cascade in apoptosis and signal transduction specifically in autism. CASP3 is one of the top ten interacting genes for both Mn and Cd.

Cellular Response: Rai et al. (2010) showed that astrocytes treated with As, Cd, and Pb triggered release of Ca^{2+} and ROS generation, resulting in apoptosis of the mixture treated astrocytes greater than would have been predicted by the individual metals. In addition, these authors observed a dose dependent decrease in the level of glial fibrillary acidic protein (GFAP) that persisted until adulthood.

At the cellular level, combinations of these metals have been shown to interact directly with Na^{+}/K^{+}-ATPase activity, typically exerting inhibitory effects. The Na^{+}/K^{+}-ATPase enzyme pumps sodium out of

cells, while pumping potassium into cells and is located in the plasma membrane of all animal cells. This enzyme plays a vital role in linking the extracellular signals to the intracellular medium in neural tissues. Toxicological studies show associations between disruptions in Na^+/K^+-ATPase, neurotransmitter release, and performance on developmental tests in animals. Antonio et al. (2002) observed significant inhibition of Na^+/K^+-ATPase activity *in vivo* following perinatal exposure of Pb and Cd, while Carfagna et al. (1996) noted greater than additive action of Pb or Cd in the presence of Mn, and Hussain et al. (1997) noted the same for Pb and Mn in rat synaptosomes *in vitro*. However, Chandra et al. (1984) in an *in vitro* study of rat synaptosomes noted only additive interactive effect of Pb, Mn and Cd with no effect of Mn, and Lai et al. (1980) noted no effects at all of Cd, Mn, and Al exposures.

Several toxicological studies have shown an association between exposure to mixtures of metals and effects on biogenic amines and neurotransmitter release. Mining waste containing a mixture of Pb, Mn and As decreased dopaminergic transmitter following depolarization (Rodriguez et al. 1998). Similarly, Mejía et al. (1997) demonstrated greater than additive changes in monoaminergic neurotransmitter levels in rat brains following administration of Pb and As. Leret et al. (2003) found similar results in a study of pregnant rats exposed to Pb and Cd, although the design of this study was such that it was not possible to determine whether the interaction was greater than additive or not. Chandra et al. (1984) in an *in vitro* study of rat synaptosomes found an interaction between Mn and Pb or Cd and dopamine and noradrenaline uptake. Shukla and Chandra (1984) observed increased dopamine and norepinephrine, no change in serotonin, and a decrease in 5-hydroxyindoleacetic acid in rats exposed to Mn, Zn, Hg and Cd.

As, Pb, and Mn have been documented to independently downregulate the expression of tyrosine hydroxylase (TH), necessary for catalyzing the first step in the biosynthesis of catecholamines.

Organ Response: Disruptions in neuronal signaling and function resulting from changes in neurotransmitter levels. Pb and Cd have been shown to cause oxidative stress in astrocytes (Lee et al., 2006; Yang et al., 2008). Pb and Cd have been shown to interfere with calcium signaling (Rai et al., 2010) and neuroendocrine controls such as thyroid hormone (Ishitobi et al., 2007; Lamb et al., 2008). Studies in rodents have shown that co- exposure to Pb and Cd affects the hypothalamic–pituitary–hepatic axis (Pillai et al., 2002, 2003), induces alterations in lipid peroxidation and modifies the ultrastructure of the brain (Zhang et al., 2009). Pb and Mn have been found to negatively affect mitochondrial function, signal transduction pathways and gene transcription (Lidsky and Schneider, 2003). Prenatal exposure to Cd

during neurulation results in a higher incidence of neural tube defects (Robinson et al. 2009), due to disturbances in cell-cycle pathways and apoptotic pathways. Exposure to Cd is associated with a decrease in the levels of growth hormone, insulin-like growth factor 1 and its binding protein, and insulin-like growth factor binding protein-3 (Lafuente et al. 2003; Turgut et al. 2005).

Organism Response: A number of toxicological studies have shown associations between disruptions in neurotransmitter levels and behavioral and motor tests, supported by epidemiologic studies.

Molecular Initiating Event: Andrade et al. (2013) found rats administered As, Pb, and Mn showed a significant ($p < 0.05$) correlation between increased Pb, As, Mn and delta-ALA levels in the brain and decreased motor activity. Urinary delta-ALA concentrations were higher in the mixture-treated group than the sum of the delta-ALA urinary levels in each single-treated groups and discriminated ($p < 0.05$) between the mixture and untreated rats. Moreover, urinary delta-ALA was correlated ($p < 0.05$) with brain delta-ALA levels. Delta-ALA is the first precursor in heme synthesis and a known biomarker of lead poisoning.

Independently, exposures to As, Mn, Pb, and also Cd are known to activate HMOX1, an essential enzyme in heme catabolism.

Cellular Response: Circulating levels of delta-ALA in both the brain and urine were increased (Andrade et al. 2013). Delta-ALA has been shown to be toxic to cultured neurons and glia at concentrations as low as 10 µM, and exposure of rat brain neuron cultures to delta-ALA has been shown to inhibit Na^+/ K^+-ATPase activity (Russell et al. 1983), in addition to the direct effect on Na^+/ K^+-ATPase activity by exposure to mixtures of metals . Ionic homeostasis maintained by the Na^+, K^+-ATPase is critical for cell growth, differentiation, and cell survival, and a deficiency has been identified as contributing to the regulation of apoptosis of astrocytes and neuronal cells (Yu 2003). Failure of the Na^+/K^+-ATPase pump results in depletion of intracellular K+, accumulation of intracellular Na+, membrane depolarization and increases in intracellular free Ca^{2+}, and has been identified in chronic neurodegenerative diseases.

Organism Response: A number of toxicological studies have shown associations between disruptions in neurotransmitter levels and behavioral and motor tests, supported by epidemiologic studies.

Table 2: Proposed Adverse Outcome Pathways for Mixtures of Metals

Adverse Effect: General Learning/Cognition Disorders as Evidenced by Lower Performance on Standard Developmental Tests in Humans

Event	Event	Genes in Common to All Four Metals	Experimental Evidence for Individual Chemical Exposure	Experimental Evidence for Combined Exposure	Experimental Evidence Against Interactive Effects
Molecular Initiating Event	Phosphorylation of MEK1/2, ERK	BDNF, CASP3, MAPK1, MAPK3	Stansfield et al. 2012; Kozul et al. 2009	Rai et al. 2010;	
Cellular Response	Increased cellular Ca^{2+}	CASP3	Stansfield et al. 2012; Kozul et al. 2009	Rai et al. 2010;	
Cellular Response	Apoptosis of astrocytes	CASP3	Stansfield et al. 2012; Kozul et al. 2009; Rocha et al. 2011	Rai et al. 2010;	
Cellular Response	ROS and oxidative stress	NOS2, CAT	Verity 1999; Aschner and Aschner 1999; Deth et al. 2008; Costa et al. 1997	Rai et al. 2010; Lee et al. 2006; Fowler et al. 2004	
Cellular Response	Catecholamine biosynthesis	TH			
Cellular Response	Neurotransmitter release; biogenic amines	TH, BDNF	Antonio et al. 2002; Leret et al. 2003; Rodriguez et al. 1998; Lai et al. 1981	Chandra et al. 1981; Shukla and Chandra 1982	
Cellular Response	Inhibition of Na^{+}/K^{+}-ATPase and Mg^{2+}-ATPase		Antonio et al. 2002	Carfagna et al. 1996; Hussain et al. 1987	Lai et al. 1980 (Cd,Mn,Al); Chandra et al. 1984 (Pb,Mn,Cd); Pillai et al. 2002 (Cd,Pb)
Organ Response	Disruptions in neuronal function	BDNF, CASP3		Rai et al. 2010;	
Organism Response	Reduced performance on behavioral, learning and development tests (e.g., WISC)		Antonio et al. 2002; Leret et al. 2003	Rai et al. 2010; Claus-Henn et al. 2012; Kim et al. 2013; Marlowe et al. 1985a; Lin et al. 2013; Thatcher et al. 1982; Wright et al. 2006	Betharia and Maher 2012 (Pb,Mn); Lucchini et al. 2012 (Pb,Mn); Wasserman et al. 2011 (Mn,As)

Population Response	Population shifts in IQ, increased incidence of learning disabilities; slight evidence for autism, ADHD				

Adverse Effect: General Learning/Cognition Disorders as Evidenced by Lower Performance on Standard Developmental Tests in Humans

Molecular Initiating Event	Transketolase with thiamine pyrophosphate uptake		Zheng 2001		
Cellular Response	Mitochondrial dysfunction			Zhang et al. 2009	
Cellular Response	ROS and oxidative stress	NOS2, CAT	Verity 1999; Aschner and Aschner 1999; Deth et al. 2008; Costa et al. 1997	Rai et al. 2010; Lee et al. 2006; Fowler et al. 2004	
Organism Response	Reduced performance on behavioral, learning and development tests (e.g., WISC)			Claus-Henn et al. 2012; Kim et al. 2013; Marlowe et al. 1985a; Lin et al. 2013; Moon et al. 1985; Thatcher et al. 1982; Wright et al. 2006	Betharia and Maher 2012 (Pb,Mn); Lucchini et al. 2012 (Pb,Mn); Wasserman et al. 2011 (Mn,As)
Population Response	Population shifts in IQ, increased incidence of learning disabilities; slight evidence for autism, ADHD				

Molecular Initiating Event	Decreased heme formation stimulating ALAS	HMOX1	Andrade et al. 2013	Andrade et al. 2013;	
Cellular Response	Heme biosynthesis	HMOX1, CAT		Andrade et al. 2013; Jadhav et al. 2007; Mahaffey and Fowler 1977; Mahaffey et al. 1981	
Organ Response	Increased delta-ALA levels in blood and urine	HMOX1	Costa et al. 1997	Andrade et al. 2013;	

Organism Response	Reduced performance on behavioral, learning and development tests (e.g., WISC)			Andrade et al. 2013; Claus-Henn et al. 2012; Kim et al. 2013; Marlowe et al. 1985a; Lin et al. 2013; Moon et al. 1985; Thatcher et al. 1982; Wright et al. 2006	Betharia and Maher 2012 (Pb,Mn); Lucchini et al. 2012 (Pb,Mn); Wasserman et al. 2011 (Mn,As)
Population Response	Population shifts in IQ, increased incidence of learning disabilities; slight evidence for autism, ADHD				

7.0 CONCLUSIONS AND DISCUSSION

1. There is evidence that Mn increases distribution or retention of Pb and Cd in the brain in a greater than additive manner, less so for As.

2. There is toxicological evidence that Mn interacts with Pb and Cd to greater than additively inhibit Na^+/K^+-ATPase activity directly, leading to increases in Ca^{2+}, increases in ROS and oxidative stress with concomitant disruptions in neurotransmitter release and neuronal function. Both Claus Henn et al. (2012) and Kim et al. (2009) found an interaction between high levels of Mn and Pb exposures in epidemiologic studies of children.

3. There is evidence of interactions leading to disruptions in heme biosynthesis as shown by increased brain and urinary delta-ALA levels in mixture-exposed animals. Significant interaction with iron, iron metabolism

4. The mixture of Pb, As and Cd show a greater than additive effect on apoptosis of astrocytes (via ERK/JNK signaling, increase in Ca^{2+} and ROS); astrocytes are a source of neurotrophic factors (BDNF implicated across all three of these constituents from the comparative toxicogenomics database); reduced glial fibrillary acidic protein expression GFAP interferes with astrocyte production, and modulates synaptic efficiency in the central nervous system.

5. A number of polymorphisms exist that impact uptake of metals, including CCL2 for As, HMOX1 for As, Pb (key heme oxygenase), NOS3

6. There are several plausible target genes in common to As, Pb, Cd and Mn, or related pathways that could act synergistically to influence exposure and effect.

Overall, there is evidence that prenatal and perinatal exposures to As, Pb, Cd, and Mn could lead to observed decreases in performance on a battery of both behavioral and cognitive tests. There is also evidence of potential antagonistic effects for selected exposures (Pb and Cd).

It is clear that evaluating exposures on an individual chemical basis does not adequately account for the wide array of mixtures encountered in the environment (Suk et al. 2002; Sargiannis and Hansen 2012). Risk assessment methods need to evolve to account for complexity in exposures and responses given

gene-environment interactions and potentially susceptible subpopulations (Yang and Dennison 2007; Gennings and Carter 1997). This systematic review documents the potential for greater than additive responses to exposure to mixtures of metals and developmental health outcomes in children.

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Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Target Organ	Exposure	N	Outcome	Methods
***in vitro* studies**					
Ben Fredj et al. 2010	Chinese hamster ovary cells transfected with heat-shock protein 47 promotor	Ni, Cd, Pb	NA	Stress response	Ternary diagram
Carfagna et al. 1996	Male Sprague-Dawley rat brain synaptic plasma membranes	Pb^{2+}, Mn^{2+}, Cd^{2+}	NA	Inhibition of Na^{+}/K^{+}-ATPase and Mg^{2+}-ATPase	Dixon plot; ANOVA
Chandra et al. 1984	Rat brain synaptosomes	Pb^{2+}, Mn^{2+}, Cd^{2+}	NA	Inhibition of Na^{+}/K^{+}-ATPase and Mg^{2+}-ATPase activities; uptake of labelled dopamine	ANOVA
Gennings et al. 2002	Cytotoxicity in human keratinocytes (NHEK)	As, Cd, Cr, Pb	2,500 cells/cm^2	Cytotoxicity	Response surface
Haldsrud and Krokje 2009	DNA double-strand breaks following high exposure in H4IIE rat hepatoma cell line	Cd, Cu, Zn	not given	DNA damage	Mann–Witney test
Hussain et al. 1987	Pretreated rat striatal synaptosomes	Pb^{2+}, Mn^{2+}	30	Monoamine uptake and Na^{+} and K^{+}-ATPase	ANOVA
Kalia et al. 1984	Brain protein from rats	Pb, Mn	NA	protein binding	
Lai et al. 1980	Rat brain synaptosomal fraction, 4 min incubation time in shaking water bath	Cd^{2+}, Mn^{2+}, Al^{3+}	16	Monoamine uptake and Na^{+} and K^{+}-ATPase	t-test

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Target Organ	Exposure	N	Outcome	Methods
Lai et al. 1981	Purified synaptosomes from the forebrain and striatum of adult male rats using sucrose-Ficoll gradients, 4 min incubation	Cd^{2+}, Mn^{2+}, Al^{3+}	16	Biogenic amine uptake	t-test
Maier et al. 2000	Mouse hepatoma Hepa-1 cells	Cd, Cr, As	NA	Biologic responses to AhR ligands	ANOVA
Tully et al. 2000	Human HepG2 cells	As, Cd, Cr, Pb	NA	Changes in patterns of gene expression and transcriptional activation in 13 signal transduction pathways	CAT-Tox(L) assay; ANOVA, Dunnett's
***in vivo* studies**					
Andrade et al. 2013	Male Wistar rats exposed to As via drinking water for 8 days; Pb and Mn via intraperitoneal injection (8 doses)	Pb, As, Mn	30	Open-field spontaneous motor activity; urinary delta-ALA	Mann-Whitney; spearmans; ROC curves
Antonio et al. 2002	Pregnant Albino rats exposed via drinking water through gestation and lactation	Pb, Cd	24 pups	Changes in motor activity, biogenic amines in the brain, Na^{+}/K^{+}-ATPase	ANOVA
Betharia and Maher 2012	Pregnant Sprague-Dawley rats *ad libitum* access to drinking water solutions from gestation through lactation and weaning	Pb, Mn	24 dams; number of pups unknown	Learning and memory based on Morris water maze test	ANOVA, Tukey's
Bizarro et al. 2003	Inhalation of CD-1 mice 1 hour twice a week for four weeks	Pb, Cd	72	Mitochondrial ultrastructure of Sertoli cells	ANOVA

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Target Organ	Exposure	N	Outcome	Methods
Chandra et al. 1980	Male ITRC mice treated intraperitoneally for 40 days	Mn, Fe, Cu	120 divided into 6 groups	ADME; Metal absorption	atomic absorption spectrophotometer
Chandra et al. 1981	Male Albino rats exposed to Mn in drinking water and intraperitoneally to lead acetate	Pb^{2+}, Mn^{2+}	8 groups of 15 each	Changes in motor activity and biogenic amines in the brain	t-test
Chandra et al. 1983	Rats exposed intraperitoneally on day 0 of gestation	Pb, Mn	40 female, 10 male	Brain growth and biochemicals in pups	t-test
Elsenhans et al. 1987	Sprague-Dawley rats exposed to binary combinations via the diet	As, Cd, Ni, Pb, Cu, Fe, Mn, Zn	28	distribution in the body	Spearman correlation coefficient
Fairhall and Miller 1941	White rats exposed orally	Pb, As	177	mortality, weight loss, food refusal, diarrhea, poor posture, gait, tissue distribution	NA
Fowler et al. 2004	Sprague-Dawley rats exposed to drinking water at 30, 90, 180 days	Pb, Cd, As	168	Molecular parameters of oxidative stress in renal and hematopoietic systems	Factorial design
Gu et al. 2008	Intragastrical perfusion of Sprague-Dawley rats once a day for six weeks	Pb, Cd	24	Divalent metal transporter 1 (DMT1)	ANOVA, t-test

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Target Organ	Exposure	N	Outcome	Methods
Hochadel and Waalkes 1997	Adult male Fischer (F344/NCr) rats exposed via one dorsal thoracic (Cd) or lumbosacral (As) injection	Cd, As	39 mice (As pretreatment); 82 mice (Cd pretreatment)	Metallothionein, hepatotoxicity, testicular necrosis	Fisher, ANOVA, Dunnett's
Jadhav et al. 2007	Male Wistar rats exposed via drinking water for 30, 60, 90 days	As, Cd, Pb, Hg, Cr, Ni, Mn, Fe	5 groups of 10 each	oxidative stress in erythrocytes	ANOVA, Duncan's *post-hoc*
Lal et al. 1980	Male Albino rats exposed intraperitoneally for 30 days; pairwise exposure (Mn + one other)	Mn^{2+}, Zn^{2+}, Hg^{2+}, Cd^{2+}	8 groups of 10 each	tissue distribution	t-test
Leret et al. 2003	Pregnant Wistar rats exposed via drinking water	Cd acetate; Pb acetate	24 pups	Behavior, biogenic amines	ANOVA
Mahaffey and Fowler 1977	Diet exposed male Sprague-Dawley rats	Pb, Cd, As	168	Blood chemistry, tissue distribution	Factorial design
Mahaffey et al. 1981	Diet exposed male Sprague-Dawley rats	Pb, Cd, As	168	Blood chemistry, tissue distribution	Factorial design
Malhotra et al. 1984	Fe-deficient male Albino rats exposed intraperitoneally (Pb) and drinking water (Mn)	Pb^{2+}, Mn^{2+}	80	Lipid peroxide formation; contents of metals in the brain	ANOVA
Mejía et al. 1997	Male BALB/c mice exposed via gastric intubation over 14 days	Pb, As		Levels of dopamine, norepinephrine, seratonin (DA, HE) and metabolites (DOPAC, f-HIAA)	ANOVA, Tukey's HSD, t-test

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Target Organ	Exposure	N	Outcome	Methods
Nampoothiri and Gupta 2008	Charles female rats treated subcutaneously	Pb, Cd	4 groups of 12-13 rats	Biochemical effects on reproductive performance, placenta, and ovary	ANOVA
Nation et al. 1990	Adult male Sprague-Dawley rats exposed via diet for 60 days	Pb, Cd	32	Behavioral and motor outcomes	Behavior, ANOVA
Pillai et al. 2002	Adult female Charles Foster rats treated intraperitoneally	Pb, Cd	unknown	Pituitary membrane, N^+/K^+-ATPase	ANOVA
Pillai et al. 2003	Adult female Charles Foster rats treated intraperitoneally	Pb, Cd	unknown	Hypothalamic-pituitary axis	ANOVA
Pillai et al. 2009	Adult female Charles Foster rats administered subcutaneously through gestation through postnatal day 21	Pb, Cd	unknown	Hepatic xenobiotic metabolizing activities	ANOVA, Bonferroni's multiple comparison test or t-test
Pillai et al. 2010	Gestational and lactational exposure in postnatal rats on ovarian steroidogenesis	Pb, Cd	unknown	Effects on ovarian steroidogenesis	ANOVA, Bonferroni's multiple comparison test or t-test

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Target Organ	Exposure	N	Outcome	Methods
Rai et al. 2010	Gavage to pregnant and lactating Wistar rats and postweaning pups up to 2 months	As, Cd, Pb	30 animals in 9 groups	glial damage and behavioral aberrations during rat brain development	ANOVA, Student-Newman
Rodriguez et al. 1998	Dopamine release from 60 adult male Wistar rats orally and subchronically exposed to mining waste	As, Mn, Pb, Cd	60	Basal dopamine release under long-standing depolarization through high-potassium perfusion	*in vivo* microdialysis; ANOVA, Tukeys HSD
Shukla and Chandra 1982	Male Albino rats exposed intraperitoneally for 30 days	Mn^{2+}, Zn^{2+}, Hg^{2+}, Cd^{2+}	8 groups of 20 animals	Biogenic amines	
Shukla and Chandra 1987	Pb in drinking water with intraperitoneal Mn or Cd administered to male Albino rats for 30 days	Pb, Mn, Cd	300	Contaminant distribution in the bodies of growing rats	ANOVA Duncan's Multiple range test

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Target Organ	Exposure	N	Outcome	Methods
Smith et al. 2012	Pb and Cd in soil administered in feed for 18 days	Pb, Cd	9	Contaminant concentrations in coexposed pregnant and nonpregnant mice	ANOVA
Zhang et al. 2009	Female Sprague-Dawley rats exposed via drinking water throughout pregnancy and pups throughout lactation	Pb, Cd	4 groups of 12-13 rats	Lipid peroxidation and ultrastructural modifications in the brain	ANOVA
Zhu et al. 2013	Female Sprague-Dawley rats exposed via drinking water during gestation; pups through gestation and lactation	Pb acetate, Fe	55	Fe status and expression of divalent metal transporter 1 (DMT1) and ferroportin 1 (FP1) in the brain of offspring rats	ANOVA

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Conclusions	Range of Concentrations	Evidence for Interaction
***in vitro* studies**			
Ben Fredj et al. 2010	Highest stress response effect observed at high Ni and Cd for several Pb levels	Unknown	**+** at higher concentrations
Carfagna et al. 1996	Cd/Mn or Pb/Mn inhibited Na^+/K^+-ATPase synergistically; Cd/Pb inhibited additively; Cd/Pb antagonistically inhibited Mg^{2+}-ATPase; Cd/Mn or Pb/Mn additively inhibited at low Mn, inhibited antagonistically at higher concentrations	0 - 3 µM	**+, -**
Chandra et al. 1984	Pb/Cd inhibition of Na^+/K^+ additive; Mn no effect. Interaction of Mn with Pb or Cd produced inhibition on the uptake of dopamine and noradrenaline	0 - 100 µM	**+,** no effect
Gennings et al. 2002	8 to 36 uM of mixture showed an increase in cytotoxicity (synergism); 80 to 120 uM showed a decrease in cytotoxicity (antagonism)	7.7 µM As, 4.9 µM Cr, and 6.1 µM Cd, 100 µM Pb	**+** at low concentrations; **-** at high concentrations
Haldsrud and Krokje 2009	Exposure to high concentrations of Cu/Cd produced a significant increase in DNA strand breaks. High concentrations of Cu/Cd/Zn resulted in significantly lower DNA double-strand breaks.	variable	**+, -**
Hussain et al. 1987	Additive inhibition of Na^+/K^+-ATPase in untreated cells; greater than additive in pretreated Fe cells	Pb 25 µM, Mn 500 µM	**+,** no effect
Kalia et al. 1984	No competitive binding but increased binding in the mixture relative to individual	Mn 1.7 mg/L, Pb 3.25 mg/L	**+**
Lai et al. 1980	Cd and Al inhibited synaptosomal choline uptake, but did not show parallel inhibitory effects on Na-K-ATPase activity directly contradicts the ionic gradient hypothesis	Cd 363 µM, Mn 1.5 mM, Al 224 µM	did not test combination

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Conclusions	Range of Concentrations	Evidence for Interaction
Lai et al. 1981	Individual inhibitory effects on dopamine uptake by forebrain synaptosomes with differential interaction	0.1 - 100 µM	did not test combination
Maier et al. 2000	No effect of Cd, As on TCDD inducubility of Cyp1a1	variable	no effect
Tully et al. 2000	Induction of several signal transduction pathways by individual metals but no synergistic activity	As(V), at doses of 50–250 mM, Pb(II) 12–100 mM, Cd(II), at 1.25–15 mM, Cr(VI) 5–10 mM	no effect
***in vivo* studies**			
Andrade et al. 2013	Metal mixture group had higher brain levels of all three metals; mixture exacerbated motor activity with strong correlation to delta-ALA levels	Pb (5 mg/kg), As (60 mg/L), Mn (10 mg/kg) individual and in combination	**+**
Antonio et al. 2002	Reduced hemoglobin, hematocrit; Na^+/K^+-ATPase significantly reduced (normal following removal of exposure); reductions in motor activity; increased dopamine and serotonin	Gestation: Pb 32 mg/kg-d, Cd 1.02 mg/kg-d; lactation Pb 65 mg/kg-d, Cd 2.2 mg/kg-d	Not possible to determine degree of interaction
Betharia and Maher 2012	Females but not males exposed to Pb or Mn displayed behavioral deficits; no effect of mixture observed. Pharmacokinetic interaction	Pb (10 µg/ml); Mn 2 mg/ml)	no effect
Bizarro et al. 2003	Statistically significantly increased mitochondrial damage from exposure to the mixture; greater than additive; dose-time relationship	lead acetate 0.01M, cadmium chloride 0.006M or combination	**+**

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Conclusions	Range of Concentrations	Evidence for Interaction
Chandra et al. 1980	Combined administration of Mn and Fe decreased the accumulation of Mn; combined Mn and Cu increased Cu accumulation	(Mn 4 mg/kg), ferric chloride (Fe 1 mg/kg) and cupric chloride (Cu 1 mg/kg)	**+, -**
Chandra et al. 1981	Observed dose-response in impairment of learning ability following combined exposure; significant increase in brain contents of biogenic amines; magnitude of lead accumulation in the brain with simultaneous exposure	3 mg Mn^{2+}/ml water; 5.0, 8.0, 12.0 mg Pb^{2+}/kg for 14 days	**+**
Chandra et al. 1983	Pb accumulation in the brain increased significantly with combined exposure; Pb and Mn exposure during gestation significantly decreased DNA in pup brains	Pb 5.0 mg/kg; Mn 6.0 mg/kg	**+**
Elsenhans et al. 1987	Increases of renal Pb and intestinal Cd by dietary Ni, and a decrease in bone As by dietary Pb	90 ppm As, 180 ppm Cd, 365 ppm Ni, and 394 ppm Pb	**+, -**
Fairhall and Miller 1941	Calcium arsenate was most toxic, lead arsenate less, and lead carbonate least toxic. Pathologic studies showed significant changes in the kidney and spleen.	Pb 18 mg/kg-d; As 6.3 mg/kg-d	did not test combination
Fowler et al. 2004	Dynamic, time-dependent alterations in inducible oxidative stress protective systems at 180 days	Pb (25 mg/l lead acetate), Cd (10 mg/l cadmium chloride), As (5 mg/l sodium arsenite)	**+**
Gu et al. 2008	Greater than additive protein levels of DMT1 from mixture	Pb 20 mg/kg-d, Cd 2 mg/kg-d and combined	**+**

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Conclusions	Range of Concentrations	Evidence for Interaction
Hochadel and Waalkes 1997	As pre-treatment protected against Cd-induced lethality; Cd pretreatment did not alter As toxicity	22.5 µM NaAsO2/kg followed by $CdCl_2$ 10, 20, 30 µM/kg; $CdCl_2$ 3.0 µM/kg followed by 67.5, 78,8, 84.4, 90.0 µM $NaAsO_2$/kg	**-** or no effect
Jadhav et al. 2007	Effects observed only at highest doses; no effects at 1x, 10x	Pb (0.22, 2.2 22.0 mg/kg); Mn (2.026, 20.26, 202.6 mg/kg); Cd (0.098, 0.98, 9.8 mg/kg)	interaction only at the highest doses
Lal et al. 1980	Combined administration of Mn and Cd increased brain content of Cd	Mn^{2+} (4 mg/kg), Zn^{2+} (1.0 mg/kg), Cd^{2+} (0.5 mg/kg), Hg^{2+} (0.5 mg/kg)	**+**
Leret et al. 2003	Increases in anxiety-like behavior; increased hippocampal dopamine and serotonin	Gestation: Pb 32 mg/kg-d, Cd 1.02 mg/kg-d; lactation Pb 65 mg/kg-d, Cd 2.2 mg/kg-d	Not possible to determine degree of interaction
Mahaffey and Fowler 1977	Increased RBCs but reduced hemoglobin and hematocrit (Pb/Cd); no effect from Pb/As	Pb 10 mg/kg-d; As 2.5 mg/kg-d	**-**, no effect
Mahaffey et al. 1981	Increased RBCs but reduced hemoglobin and hematocrit (Pb/Cd); no effect from Pb/As	Pb 10 mg/kg-d; As 2.5 mg/kg-d	**-**, no effect
Malhotra et al. 1984	Concurrent exposure to lead and manganese increased the lipid peroxidation potential of brain in iron deficient rats	Mn^{2+} (3 mg per ml of water, orally), Pb^{2+} (8 mg/kg, i.p.)	**+**
Mejía et al. 1997	Mixture showed higher accumulation of Pb and lower As than single constituent; mixture increased DOPAC in the hypothalamus and DA and 5-HIAA in the striatum, decreased NE	Pb (74.0 mg/kg-d), As (8.0 mg/kg-d)	**+**

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Conclusions	Range of Concentrations	Evidence for Interaction
Nampoothiri and Gupta 2008	Pb and Cd alone or in combination had no significant effect on reproductive performance	0.05 mg/kg-d individually and combined	no effect
Nation et al. 1990	Cotreatment showed no behavioral differences relative to no exposure	Pb (acetate) 500 mg/kg; Cd (chloride) 100 mg/kg and combined	Not possible to determine degree of interaction
Pillai et al. 2002	Pb/Cd inhibition of Na^+/K^+ additive or less than additive	Pb (acetate) and Cd (acetate) 0.05 mg/kg daily individually; 0.025 in combination for 15 days	-
Pillai et al. 2003	Decrease in serotonin and norephinephrine; significant decrease in serum luteinizing hormone and follicle stimulating hormone; effects seen with Cd alone also. Pb seems to mediate Cd effect	Pb (acetate) and Cd (acetate) 0.05 mg/kg daily individually; 0.025 in combination for 15 days	-
Pillai et al. 2009	No significant effect on reproductive performance; inhibition of phase I and phase II enzyme activities but not synergistic	lead acetate, cadmium acetate (0.05 mg/kg-d indivdually, 0.025 mg/kg-d combined)	no effect
Pillai et al. 2010	No significant effect on reproductive performance; key steroidogenic enzymes impacted but not synergistically	lead acetate, cadmium acetate (0.05 mg/kg-d indivdually, 0.025 mg/kg-d combined)	no effect

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Conclusions	Range of Concentrations	Evidence for Interaction
Rai et al. 2010	Greater than additive downregulation in viability and increased apoptosis of astrocytes, induced by ERK/JNK signaling, increase in Ca^{2+} and ROS generation	Pb 0.22 mg/kg + Cd 0.098 mg/kg + As 0.38 mg/kg; Pb 2.22 mg/kg + Cd 0.98 mg/kg + As 3.8 mg/kg; individual groups Pb 2.22, 6.66 m/gkg, Cd, 0.98, 2.94 mg/kg, As 3.8, 11.4 mg/kg	+
Rodriguez et al. 1998	Mining waste exposed animals were not able to sustain increased dopamine release in response to depolarization. No other differences	As 9647 mg/kg; Mn 1650 mg/kg; Pb 690 mg/kg; Zn 1350 mg/kg; Cu 1180 mg/kg	Not possible to determine degree of interaction
Shukla and Chandra 1982	Increased dopamine and norepinephrine; no change in serotonin, decrease in 5-hydroxyindoleacetic acid	Mn^{2+} (4 mg/kg), Zn^{2+} (1.0 mg/kg), Cd^{2+} (0.5 mg/kg), Hg^{2+} (0.5 mg/kg)	+, no effect
Shukla and Chandra 1987	Enhanced accumulation of all three metals in the brain, Mn in liver, Pb in kidney and Cd in testis and kidney after combined exposure. Changes in the metallic distribution within the tissues after coexposure may be the result of a competition between the administered metals for common binding sites.	Pb^{2+} 5 mg/l *ad libitum*, Mn^{2+} 1 mg/kg, Mn^{2+} 4 mg/kg , Cd^{2+} 0.1 mg/kg , Cd^{2+} 0.4 mg/kg, Pb^{2+} 5 mg/l *ad libitum* with Mn^{2+} 1 mg/kg, Pb^{2+} 5 mg/l *ad libitum* with Mn^{2+} 4 mg/kg, Pb^{2+} 5 mg/l a*d libitum* with Cd^{2+} 0.1 mg/kg, Pb^{2+} 5 mg/1 *ad*	+

Table S1: Summary of Toxicological Studies Evaluating Two or More Metals

Reference	Conclusions	Range of Concentrations	Evidence for Interaction
Smith et al. 2012	Pregnant mice showed highest Pb concs, statistically significantly higher than non-pregnant. Co-administration of Cd reduced Pb concentration	Pb 736 mg/kg; Pb 736 mg/kg with Cd 20 mg/kg	-
Zhang et al. 2009	Greater than additive effect on mitochondrial swelling and other ultrastructural modifications; decrease in glutathione peroxidase, superoxide dismutase, catalse, acetylcholinesterase; incraese in maleic dialdehyde	Pb (300 mg/l); Cd (10 mg/L) and combined	+
Zhu et al. 2013	Pb exposure increases Fe content in old-aged rats' brain	510 (low lead group, LLG) or 956 mg/L lead acetate (high lead group, HLG)	did not test combination

Table S2: Summary of Epidemiologic Studies Including Two or More Metals

Reference	Study Design	N	Metals Evaluated	Exposure Measure	Exposure Levels
Adams et al. 2009	cross-sectional	63 children age 3-8 yrs	Sb, **Pb**, Sn, Tl, Hg, Wb, **As, Cd**, Al, Sn	Urine (metals measured before and after chelation with DMSA)	Means: Pb: 1.3 µg/g creatinine; As: 32 µg/g creatinine; Cd: 0.38 µg/g creatinine
Boucher et al. 2012	prospective longitudinal study	196 school-age Inuit children from Arctic Québec	PCB, **Pb,** Hg	Blood (cord and current at ~11 years old)	Means: cord Pb: 4.8 µg/dl; current Pb: 2.6 µg/dl
Calderon et al. 2001	cross-sectional	41 high exposure; 39 low exposure	**Pb, As**	Urinary As; blood Pb	GMs for exposed group: urinary As = 62.9 µg As/g creatine; blood Pb 8.9 µg/dl; for unexposed group: As = 40.2 µg/g; Pb = 9.7 µg/dl
Claus Henn et al. 2012	prospective longitudinal study	450 Mexican children	**Mn, Pb**	Blood (1 & 2 year olds)	24.7 ± 5.9 Mn= µg/L and 21.5 ± 7.4 µg/L Pb= 5.1 ± 2.6 µg/dL and 5.0 ± 2.9 µg/dL
Geier et al. 2012	cross-sectional	18 children age 1-6 yrs	**As**, Hg, **Cd, Pb**, Cr, Co, Ni, Al, U, **Mn**, Sn	Hair	µg/g: As 0.074 ± 0.058 (0.02–0.26), Pb 0.63 ± 0.50 (0.18–1.7), Hg 0.33 ± 1.01 (0.03–4.4), Cd 0.18 ± 0.14 (0.054–0.53), Cr 0.41 ± 0.11 (0.29–0.69), Co 0.03 ± 0.03 (0.003 ± 0.15), (Ni 0.17 ± 0.1 (0.05–0.46), Mn 0.4 ± 0.4 (0.08–1.7), Al 13 ± 5.8 (6.2–29), Sn 0.44 ± 0.25 (0.12–0.96), U 0.046 ± 0.035 (0.003–0.13)

Table S2: Summary of Epidemiologic Studies Including Two or More Metals

Reference	Study Design	N	Metals Evaluated	Exposure Measure	Exposure Levels
Haynes et al. 2011	ecological	88 Ohio counties	**Pb, Mn, As, Cd**, Cr, Hg	Air emissions	Pb 4.9–25,932, Mn 0.7–208,059, As 0.1–5561, Cd 0.1–681, Cr 1–24,325, Hg 0.1–799
Khan et al. 2011	cross-sectional	201 Bangladeshi Children 8–11 years of age	**Mn, As**	Tube well water	As (µg/L) 0.9–18.0, Mn (µg/L) 6.3–33.9, Pb (µg/L) 36.7–245.7
Kim et al. 2009	cross-sectional	261 Korean children aged 8–11 years.	**Pb, Mn**	Blood	mean Pb: 1.73 µg/dL (SD = 0.8; median = 1.55; range = 0.42–4.91), mean Mn: 14.3 µg/L (SD = 3.8; median = 14.0; range = 5.30–29.02)
Kim et al. 2013	prospective longitudinal study	884 women-infant pairs	**Pb, Cd**	Maternal blood (early and late pregnancy)	mean maternal blood Pb= 1.36 µg/dL (10th percentile = 0.83; 90th percentile = 2.13; range = 0.26–9.10); mean Cd=1.42 µg/L (10th percentile = 1.01; 90th percentile = 2.16; range = 0.03–9.87) during early pregnancy; 1.27 µg/dL (10th percentile = 0.77; 90th percentile = 2.10; range = 0.12–4.28) for Pb and 1.52 µg/L for Cd (10 percentile = 1.07; 90th percentile = 2.10; range = 0.43–3.73) during late pregnancy
Lee et al. 2006	cross-sectional	10,098 adult participants	**Pb, Cd**	Blood Pb, Urinary Cd, oxidative stress related markers (NHANES)	Cutoff points of blood lead deciles were 1.0, 1.5, 1.9, 2.4, 2.9, 3.5, 4.2, 5.2, and 7.1 µg/dL and those of urinary cadmium were 0.11, 0.18, 0.25, 0.33, 0.41, 0.52, 0.67, 0.88, and 1.24 µg/g creatinine.

Table S2: Summary of Epidemiologic Studies Including Two or More Metals

Reference	Study Design	N	Metals Evaluated	Exposure Measure	Exposure Levels
Lin et al. 2013	prospective cohort	230 mother-infant pairs	**Mn, Pb, As**, Hg	Cord blood	Mn= 47.90 µg/L (range,17.88–106.85 µg/L), Pb=11.41 µg/L (range0.16–43.22 µg/L), As= 4.05 µg/L (range,1.50–12.88 µg/L) and Hg=12.17 µg/L (range,1.53–64.87 µg/L)
Lucchini et al. 2012	cross-sectional	299 adolescents 11-14 yrs	**Pb, Mn**	Blood Pb, Blood/Hair/Air/Soil Mn	(BPb) 1.71 µg/dL (median1.5, range0.44–10.2),(BMn) 11.1 µg/dL (median10.9 range 4.00–24.1)
Marlowe et al. 1985a	cross-sectional	80 elementary children	**Pb, As**, Hg, **Cd**, Al	Hair	Pb 6.65 ppm, As 2.94 ppm, Hg 0.99 ppm, Cd 0.64 ppm, Al 9.41 ppm
Marlowe et al. 1985b	cross-sectional	69 elementary children	**Pb, As**, Hg, **Cd**, Al	Hair	Pb 6.65 ppm, As 2.94 ppm, Hg 0.99 ppm, Cd 0.64 ppm, Al 9.41 ppm
Moon et al. 1985	cross-sectional	69 elementary children	**Pb, As**, Hg, **Cd,** Al	Hair	Pb 6.65 ppm, As 2.94 ppm, Hg 0.99 ppm, Cd 0.64 ppm, Al 9.41 ppm
Obrenovich et al. 2011	case-control	26 children with autism spectrum disorder; 39 healthy age-matched controls	**As**, **Pb**, Hg, Cu, Fe	Hair, urine	As 0.5 mg/kg, Pb 0.5 mg/kg, Hg 0.25 mg/kg Ni 0.25 mg/kg, Mn 0.5 mg/kg
Parajuli et al. 2013	hospital-based birth cohort	100 mother-infant pairs in Nepal	**Pb, As**, Zn	Cord blood	Pb 6.83–220.8 µg/L, As 0.51–9.58 µg/L, Zn1299–6430 µg/L

Table S2: Summary of Epidemiologic Studies Including Two or More Metals

Reference	Study Design	N	Metals Evaluated	Exposure Measure	Exposure Levels
Roberts et al. 2013	nested case-control	325 cases; 22,101 controls; offspring of Nurses' Health Study participants	diesel, **Pb, Mn,** Hg, methylene chloride, metals overall	Modeled air pollution (US EPA National Air Toxic Assessment)	Air concentration specific to census tract
Rosado et al. 2007	cross-sectional	602 children 6-8 yrs	**As, Pb**, Zn, Fe	Blood Pb, urinary As	UAs concentrations> 50 µg/L, PbB ≥ 10 µg/dL, Zn ≤ 65 mg/dL, Hb was < 12.4 g/dL, and ferritin deficiency ≤ 12 µg/L and Cu deficiency when serum copper was < 80 µg/
Thatcher et al. 1982	cross-sectional	149 children 5-16 yr	**Cd, Pb**	Hair	5 - 22 ppm Pb, 0.5 - 1.3 Cd
Wasserman et al. 2011	cross-sectional	299 children 8-11 yrs	**As, Mn**	Blood	3 studies: Water As (µg/l) 117.8 3.0 120.1; Urinary As (µg/l) 116.6 57.5 110.7 Water Mn (µg/l) 1386 797 1302
Wright et al. 2006	cross-sectional	32 children 11-13 yrs	**Mn, As, Cd**	**Hair**	Mn 471.5 ppb, As 17.8 ppb, Cd 57.7 ppb

Table S2: Summary of Epidemiologic Studies Including Two or More Metals

Reference	Outcome	Interactions between toxicants assessed?	Conclusions
Adams et al. 2009	Autism, assessed with: 1) Autism Diagnostic Observation Schedule, 2) Pervasive Developmental Disorders Behaviour Inventory, 3) Autism Treatment Evaluation Checklist, and 4) Severity of Autism Scale	No	Urinary Pb (and Sb) were most strongly associated with autism severity. Authors report high correlations between metals and thus suggest interpreting results as evidence for a general role of toxic metals in relation to autism severity rather than evidence for any particular metal.
Boucher et al. 2012	Executive function and impulsivity, measured by go/no-go performance (mean reaction time, percent correct go, percent correct no-go) and five event-related potentials (N2, P3, error-related negativity, error positivity (Pe), and correct response positivity (Pc))	Yes (cross products for binary & 3-way interactions; stratified analyses split at medians)	Effects of cord Pb were seen primarily in the children with higher prenatal PCB and/or Hg exposures, indicating that the effects of prenatal Pb exposure were intensified by heavier PCB and Hg exposures.
Calderon et al. 2001	WISC-RM: full, verbal, performance IQ; long-term memory; linguistic abstraction; attention span; visuospatial organization	Yes (no results shown)	Reported that no interaction was observed (but results not shown). Verbal IQ decreased with increasing concentrations of urinary As associated with decreased verbal IQ, poorer performance on long-term memory and linguistic abstraction; Pb in blood associated with lower attention scores.
Claus Henn et al. 2012	Bayley Scales of Infant Development–II	Yes	Observed evidence of synergism between lead and manganese, whereby lead toxicity was increased among children with high manganese coexposure.
Geier et al. 2012	Childhood Autism Rating Scale (CARS)	No	Hair Hg concentrations significantly and positively correlated with increased ASD severity. In contrast, no significant correlations were observed between other hair metals and ASD severity.

Table S2: Summary of Epidemiologic Studies Including Two or More Metals

Reference	Outcome	Interactions between toxicants assessed?	Conclusions
Haynes et al. 2011	Youth adjudications for criminal activity	No	Airborne exposure to manganese, mercury, and particulate matter correlated with increased risk of adjudication.
Khan et al. 2011	Child Behavior Checklist-Teacher's Report Form (CBCL-TRF), Wechsler Abbreviated Scale of Intelligence (WASI)	Yes	No Mn-As interactions observed. Water Mn concentrations positively associated with behavior scores; blood Mn and As not associated with behavior scores.
Kim et al. 2009	Korean Educational Development Institute-Wechsler Intelligence Scales (KEDI-WISC, 1986)	Yes	Mn-Pb interaction, whereby adverse Pb effects on IQ observed only among kids with high Mn
Kim et al. 2013	Bayley Scales of Infant Development–II administered to infants at 6 months of age	Yes (stratified, split Cd at median)	Possible antagonistic interaction between Pb and Cd in maternal blood during early pregnancy for MDI score. Synergistic effect during late pregnancy, whereby adverse Pb effects on MDI and PDI were only significant at high Cd levels
Lee et al. 2006	oxidative stress markers of serum γ-glutamyltransferase (GGT), vitamin C, carotenoids, and vitamin E	No	Strong association of blood Pb and urinary Cd with oxidative stress markers

Table S2: Summary of Epidemiologic Studies Including Two or More Metals

Reference	Outcome	Interactions between toxicants assessed?	Conclusions
Lin et al. 2013	Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT)	Yes (between Mn and Pb)	Mn-Pb interaction, whereby Mn and Pb ≥ 75th percentile associated with lower scores than low Mn and low Pb; no association with As or Hg
Lucchini et al. 2012	Wechsler Intelligence Scale for Children (WISC) and the Conners-Wells' Adolescent Self-Report Scale Long Form (CASS:L)	Y**es**	No interaction. Main effects of Pb: 2.4 IQ pt reduction for a two-fold increase of blood Pb. No main effect of Mn
Marlowe et al. 1985a	Walker Problem Behavior Identification Checklist	Yes (correlation analysis)	Significant increase in explained variance due to Pb-As, Pb-Cd interactions, as well as Pb main effect
Marlowe et al. 1985b	Bender Visual Motor Gestalt Test	Yes (correlation analysis)	Combinations of As-Al, Pb-Al, As-Pb, Pb-Cd were found to account for significant proportion of variance in classroom teachers' ratings
Moon et al. 1985	Wide Range Achievement Test, Bender Visual-Motor Gestalt Test	Yes (correlation analysis)	As and interaction of As/Pb significantly associated with decreased reading and spelling; increases in Al and Al/Pb significantly associated with decreased visual-motor performance
Obrenovich et al. 2011	ASD cases compared to controls on 1) erythrocyte transkelotase, a marker of thiol metabolism, and 2) hair metals concentrations	No	Children with ASD demonstrated abnormal markers of thiol metabolism, and higher levels of hair As
Parajuli et al. 2013	Brazelton neonatal behavioral assessment scale, third edition (NBAS III)	No	Cord blood levels of Pb and As negatively associated with motor cluster scores; As inversely associated with state regulation cluster scores.

Table S2: Summary of Epidemiologic Studies Including Two or More Metals

Reference	Outcome	Interactions between toxicants assessed?	Conclusions
Roberts et al. 2013	Autism Spectrum Disorder	Evaluated overall measure of metal exposure	Perinatal exposures to the highest versus lowest quintile of diesel, lead, manganese, mercury, methylene chloride, and an overall measure of metals were significantly associated with ASD, with odds ratios ranging from 1.5 (for overall metals measure) to 2.0 (for diesel and mercury).
Rosado et al. 2007	Wechsler Intelligence Scale for Children (WISC), Cognitive Abilities Test, Peabody Picture Vocabulary Test (PPVT), curriculum-based Math Achievement Test, test of Visual-Spatial Abilities with Figure Design	Yes, but not reported	Urinary As associated with poor performance on WISC, independent of Pb
Thatcher et al. 1982	WISC-R, Wechsler Preschool and Primary Scale of Intelligence (WPPSI), Wide Range Achievement Test (WRAT), Test of Motor Impairment (MIT)	No	Hierarchical regression analyses suggest that cadmium has a significantly stronger effect on verbal IQ than does lead and that lead has a stronger effect on performance IQ than does cadmium.
Wasserman et al. 2011	Wechsler Intelligence Scale for Children-IV (WISC-IV)	Yes	Significant association between cognitive outcomes and each metal individually. No significant Mn-As interaction.
Wright et al. 2006	Wechsler Abbreviated Intelligence Scale (WASI), Wide Range Assessment of Visual Motor Ability (WRAVMA), CELF-3, Children's Category Test-Level II (CCT), California Verbal Learning Test-Children (CVLT-c), Wide Range Assessment of Memory and Learning (WRAML), Children's Depression Inventory (CDI), Behavior Assessment System for Children (BASC)	Yes	Significant interaction between highest Mn and As

Table S3: Genes in Common Across Cd, Pb, As, and Mn from www.ctdbase.org

Outcome	Pb	As	Mn	Cd
ADHD	DRD2, GRM5	LPHN3	DRD2, SLC6A3, TACR1	DRD2, DRD4, TACR1
Anxiety Disorders	MAOA	CRHR1, FOS, SLC6A4, TNF	APP, DRD2, FOS, SLC6A3, TNF, UCN	ADORA2A, APP, CRP, DRD2, FOS, MIF, NPY, SERPINA1, SLC6A4, TNF
Asperger Syndrome		SLC6A4		SLC6A4
Autism	BCL2, BDNF, CAT, CP, GABRA1, GAD1, GRIN2A, HLA-, DRB1, HRAS, IFNG, IGF1, ITGB3, JMJD1C, MAOA, MECP2, MET, NOS2, NTRK2, PON1, PRKCB, PTGS2, ROBO1, SLC1A3, SND1, TF, TNFRSF1B, XDH	ADM, BCL2, BDNF, CAT, GSTM1, GSTP1, HLA-, DRB1, IGF1, IL10, IL1RN, IL2, IL6, LAMB1, LEP, MEF2C, MTHFR, NOS2, PDE4B, PER1, PLAUR, PON1, PRF1, PTGS2, SCN7A, SERPINE1, SLC6A4, XPC	ADM, AQP4, BDNF, CAT, FOXP2, GRIN2A, IFNG, IL2, IL6, LEP, NOS2, PARK2, PTGS2, SLC1A3, TF	ADM, AR, BCL2, BDNF, CAT, DRD3, EGR2, GHR, GJA1, GPX1, GSTP1, IFNG, IGF1, IGF2, IL10, IL1RN, IL4, IL6, MET, MIF, MTF1, NOS2, PER1, PLA2G4A, PLAUR, POMC, PRL, PTGS2, SCT, SEMA5A, SERPINE1, SLC40A1, SLC6A4, TF
Child development disorders, pervasive	GRIN2B, HFE, ITGB3, LAMC3, MECP2	FOXP1, MEF2C, SOX9	GRIN2B, HFE	DRD4
Cognition disorders	APOE, APP, DRD2, MET, PTGS2, SLC1A1	APOE, MT1, MT2, PTGS2, SLC6A4	APP, DRD2, EPO, PTGS2, SLC4A10	APP, DRD2, DRD3, EPO, IGF2, MET, MT1, MT2, PTGS2, SLC6A4
Intellectual disability	BDNF, GAMT, GNAS, GRIN2B, MECP2	BDNF, MEF2C	BDNF, GRIN2B, SLC4A10	BDNF, FGFR2
Iron metabolism disorders	CP, HMOX1	HMOX1, SLC11A2, SOD1, TNF	HMOX1, TFRC	BMP2, FTH1, FTL, HAMP, HMOX1, SLC11A2, SLC40A1, SOD1, TF, TFNC, TNF
Learning Disorder	ACHE, APP, BCL2, CASP3, HMOX1, IL1B, MAPT, MECP2, TH, TRH	ACHE, BCL2, CASP3, HMOX1, HTR1A,IL1B, MAPT, MT1, MT2, TH, VEGFA	APP, CASP3, HMOX1, HTR1A, IL1B, PARK2, TH, VEGFA	ACHE, APP, BCL2, CASP3, HMOX1, IL1B, MT1, MT2, TH, VEGFA
Memory disorders	ACHE, APP, BCL2, HTR6, IL1B, MAPT			

Notes:

Red - gene in common across all four metals; blue - gene in common across three metals; green - gene in common across two metals

Table S4: Genes In Common Across Endpoints Within a Chemical

Outcome	Pb	As	Mn	Cd
ADHD	DRD2, GRM5	LPHN3	DRD2 , SLC6A3 , TACR1	DRD2 , DRD4 , TACR1
Anxiety Disorders	MAOA	CRHR1, FOS, SLC6A4, TNF	APP , DRD2 , FOS , SLC6A3 , TNF , UCN	ADORA2A , APP , CRP , DRD2 , FOS , MIF , NPY , SERPINA1 , SLC6A4 , TNF
Asperger Syndrome		SLC6A4		SLC6A4
Autism	BCL2, BDNF, CAT, CP, GABRA1, GAD1, GRIN2A, HLA-, DRB1, HRAS, IFNG, IGF1, ITGB3, JMJD1C, MAOA, MECP2, MET, NOS2, NTRK2, PON1, PRKCB, PTGS2, ROBO1, SLC1A3, SND1, TF, TNFRSF1B, XDH	ADM, BCL2, BDNF, CAT, GSTM1, GSTP1, HLA-, DRB1, IGF1, IL10, IL1RN, IL2, IL6, LAMB1, LEP, MEF2C, MTHFR, NOS2, PDE4B, PER1, PLAUR, PON1, PRF1, PTGS2, SCN7A, SERPINE1, SLC6A4, XPC	ADM , AQP4 , BDNF , CAT , FOXP2 , GRIN2A , IFNG , IL2 , IL6 , LEP , NOS2 , PARK2 , PTGS2 , SLC1A3 , TF	ADM , AR , BCL2 , BDNF , CAT , DRD3 , EGR2 , GHR , GJA1 , GPX1 , GSTP1 , IFNG , IGF1 , IGF2 , IL10 , IL1RN , IL4 , IL6 , MET , MIF , MTF1 , NOS2 , PER1 , PLA2G4A , PLAUR , POMC , PRL , PTGS2 , SCT , SEMA5A , SERPINE1 , SLC40A1 , SLC6A4 , TF
Child development disorders, pervasive	GRIN2B, HFE, ITGB3, LAMC3, MECP2	FOXP1, MEF2C, SOX9	GRIN2B, HFE	DRD4
Cognition disorders	APOE, APP, DRD2, MET, PTGS2, SLC1A1	APOE, MT1, MT2, PTGS2, SLC6A4	APP , DRD2 , EPO , PTGS2 , SLC4A10	APP , DRD2 , DRD3 , EPO , IGF2 , MET , MT1 , MT2 , PTGS2 , SLC6A4
Intellectual disability	BDNF, GAMT, GNAS, GRIN2B, MECP2	BDNF, MEF2C	BDNF , GRIN2B , SLC4A10	BDNF , FGFR2
Iron metabolism disorders	CP, HMOX1			
Learning Disorder	ACHE, APP, BCL2, HMOX1, IL1B, MAPT, MECP2, TH, TRH	ACHE, BCL2, HMOX1, HTR1A, IL1B, MAPT, MT1, MT2, TH, VEGFA	APP , HMOX1 , HTR1A , IL1B , PARK2 , TH , VEGFA	ACHE , APP , BCL2 , HMOX1 , IL1B , MT1 , MT2 , TH , VEGFA
Memory disorders	ACHE, APP, BCL2, HTR6, IL1B, MAPT			

Notes:

Red - gene in common across four or more endpoints within a metal; blue - gene in common across three endpoints;
green - gene in common across two endpoints