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Exposure to Mixtures of Metals and Neurodevelopmental Outcomes: A Review

Katherine von Stackelberg^{1,2}, Elizabeth Guzy², Tian Chu², and Birgit Claus Henn^{2,3}

¹ Harvard Center for Risk Analysis, Boston, MA 02215; kvon@hsph.harvard.edu

² Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA 02215

³ Now at the Department of Environmental Health, Boston University School of Public Health, Boston, MA 02118

Abstract

Current risk assessment guidance calls for an individual chemical-by-chemical approach that fails to capture potential interactive effects of exposure to environmental mixtures in the context of genomic influences. As an exploratory analysis, we develop a systematic review of prenatal and perinatal exposure to mixtures of lead (Pb), arsenic (As), cadmium (Cd), and manganese (Mn) and nonspecific developmental outcomes in children, including reduced IQ and decreased performance on developmental tests, using an adverse outcome pathway (AOP) framework to integrate lines of evidence based on evolving guidance developed by the Organization for Economic Cooperation and Development (OECD). Toxicological evidence suggests a greater than additive effect of combined exposures to As-Pb-Cd and Mn in the presence of one or more metals. Several epidemiologic studies also suggest synergistic effects from binary combinations of Pb-As and Pb-Cd. However, monitoring data from the National Health and Nutrition Examination Survey (NHANES) reveals the majority of the U.S. population has biomarker levels below those corresponding to exposure concentrations from the epidemiologic studies. This systematic review integrates relevant and diverse data sources using an AOP framework and provides an initial application of the OECD guidance in the context of potential neurodevelopmental toxicity of several metals, recognizing the evolving nature of regulatory interpretation and acceptance.

Keywords

Arsenic; lead; manganese; cadmium; developmental; metal mixtures; systematic review; adverse outcome pathway

1.0 INTRODUCTION

The regulatory science of risk assessment informs 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 reflecting true environmental exposures. As our understanding of complex exposures and interactions continues to grow, 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.

Environmental exposures are virtually always to complex mixtures, and while mathematically characterizing dose additivity is relatively straightforward, potential synergistic or antagonistic interactions present significant statistical challenges due to the large number of predictors and interactions, potential correlations, non-linear dose-response functions, and likely effect modification within the mixture⁽¹⁾. Nonchemical stressors (e.g., nutritional and socioeconomic status) also impact potential responses to chemical mixtures, and genetic polymorphisms and epigenetic changes are likely to influence outcomes. Cumulative risk assessment methods and approaches are evolving to meet regulatory needs⁽²⁻⁴⁾, but significant challenges remain.

The Superfund Research Program at the Harvard School of Public Health (www.srphsph.harvard.edu) explores these issues in the context of prenatal and perinatal exposures to mixtures of As, Pb, and Mn and developmental outcomes in children. As and Pb rank first and second, respectively, on the ATSDR 2011 substance priority list at National Priorities List (NPL) sites based on a combination of frequency, toxicity, and potential for human exposure at NPL sites, while Cd ranks 7th and Mn 140th. The association between developmental health outcomes in children and exposure individually to Pb⁽⁵⁾, Mn⁽⁶⁾, As⁽⁷⁾ and Cd⁽⁸⁾ is increasingly well-established. In general, the assessment of developmental outcomes is relatively non-specific (e.g., poor performance on IQ and other developmental tests) although epidemiologic evidence is growing for associations with specific cognitive outcomes such as autism and lead⁽⁹⁾ and Attention-Deficit Hyperactivity Disorder (ADHD) and both Mn⁽¹⁰⁾ and Pb^(11, 12). Evidence for the potential impact of combined exposures across two or more metals is less well understood⁽¹³⁻¹⁵⁾. In addition, the role that the genome might play in interacting with exposures to influence metal metabolism and uptake⁽¹⁶⁻²¹⁾ to result in observed health outcomes^(12,20,21) provides additional support for gene-environment interactions contributing to observed variability and potential susceptibility in population responses.

The Agency for Toxic Substances and Disease Registry (ATSDR) has developed “interaction profiles” for several chemicals including Pb, Cd, chromium (Cr), and As^(22, 23) based on a binary weight-of-evidence approach^(24,25), leading to 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 Pb and As for developmental outcomes, and possible evidence for a 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 potential interactions between two constituents. There are no published studies that extend the analysis to combinations of three and four constituents simultaneously.

1.1 Purpose of the Review

Given the potential for exposures to individual metals to result in adverse neurodevelopmental outcomes, the purpose of our systematic review is to evaluate the

evidence for potential exposures to concentrations of mixtures of metals in conjunction with genetic influences to result in neurodevelopmental health outcomes of greater or lesser magnitude than would be predicted from exposures to individual metals alone. Neurodevelopmental outcomes encompass a range of cognitive and behavioral deficits as evidenced by reduced performance on standard developmental tests specific to a particular age group with established psychometric properties that form the basis of most epidemiologic studies⁽²⁶⁾. Similarly, there are analogs to developmental tests in humans for rodents that facilitate interpretation of toxicological studies. As shown in Figure 1, the review focuses on the intersection of domains of evidence ranging from outcome-focused epidemiologic studies to mechanistic-based toxicological studies, environmental exposures (environmental exposure concentrations relative to biologically-effective doses), and what is known about cellular events at the subclinical level in relation to neurodevelopmental health outcomes. We evaluate biological plausibility with respect to hypothesized modes-of-action based on the best available understanding of molecular events, cellular targets, and genomic interactions required for neurodevelopmental effects relevant to learning and cognition to occur in the context of normal neuronal development and function using an adverse outcome pathway (AOP) framework. The AOP is a conceptual framework as shown in Figure 2 for organizing and evaluating evidence for predictive and/or causal linkages between observable, and ideally measurable, biological changes starting with a molecular initiating event (MIE) through a series of cellular and organ-level events (referred to as key events; KEs, which are typically supported through evidence from toxicological studies) essential to the progression to an adverse outcome (AO) considered relevant to regulatory decision making. Finally, biomonitoring studies and data together with exposure levels from the epidemiologic and toxicological studies provide an indication of the implications to public health more broadly.

2.0 Methods

2.1 Literature Search

We conducted a literature search to develop a database of studies categorized by exposure, toxicology, epidemiology, gene-environment, and health outcome. The literature search relied on standard search engines including PubMed, Web of Science, Google Scholar, Environmental Index, Scopus, Toxline, and Toxnet. The search strategy included terms related to metal mixtures, individual metals (e.g., arsenic, lead, manganese, cadmium), neurodevelopmental health outcomes, and associated Medical Subject Headings (MeSH) terms. We reviewed references from individual citations to identify additional studies not captured through the literature search itself.

The Comparative Toxicogenomics Database (available at www.ctdbase.org) was used 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. Genes and pathways were cross-referenced and summarized based on information from the National Center for Biotechnology Information gene database (available at www.ncbi.nlm.nih.gov/gene/).

To explore potential exposures, we extracted biomonitoring data from the National Health and Nutrition Examination Survey (NHANES, available from <http://www.cdc.gov/nchs/nhanes.htm>) for the general population to compare to exposure levels from the epidemiologic studies for urinary and blood levels of As, Cd, and Pb using the cohorts as described in the epidemiologic studies (e.g., women 16-45, and so on) to explore exposure levels in the United States. Exposure data for Mn, not available through NHANES, were obtained from Rice et al.⁽¹⁰⁹⁾ as described in the results section.

2.2 Database development

We first categorized each study for inclusion in separate databases (e.g., epidemiologic, toxicological, exposure, 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.

Epidemiologic—Each study was reviewed by one of the authors and relevant data extracted including study design and number in cohort (e.g., cross-sectional, prospective cohort, etc.), exposure measures and outcomes, tests for interactions, key conclusions, statistical approaches, and novel concepts.

Toxicological—Each study was reviewed by one of the authors and relevant data extracted including study type (e.g., *in vivo*, *in vitro*, etc.), study design (e.g., gestational, reproduction etc.), number of individuals, exposure levels, target tissues/organs and responses, dosing vehicle (e.g., gavage, oral), statistical tests, tests for interactions, and key conclusions.

An additional separate database was developed for common genetic targets identified from the Comparative Toxicogenomics Database focusing on those genes with documented impacts on learning, development, cognition, and specific developmental outcomes such as ADHD and autism. The database includes information on genetic targets, gene functions (from the National Center for Biotechnology Information gene database), and reported impact of the individual chemical exposure (e.g., activation, inhibition).

2.3 Analysis

We first evaluated toxicological studies 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 data quality for each study. We further evaluated each study for evidence of greater or less than additive effects with respect to the outcome being studied, and as to how and where study results might fit in an adverse outcome pathway based on the outcome (e.g., molecular initiating event, key event at the cellular level, key event at the organ or organism level) as defined by guidance from the Organization for Economic Cooperation and Development (OECD)⁽²⁷⁻³⁰⁾. Epidemiologic studies were similarly evaluated with respect to data quality and study design, followed by an evaluation of the evidence for or against synergistic effects. Where possible, we

compared *in vivo* doses or body burdens from the epidemiologic studies to NHANES data or other sources.

Finally, we integrated these data using an AOP framework to evaluate the evidence by adapting the modified Bradford Hill considerations as presented in OECD guidance⁽³⁰⁾. As shown in Figure 2, the AOP identifies a series of key events (KEs) essential to the progression from a molecular initiating event (MIE) to an adverse outcome. Predictive and/or causal linkages between KEs are referred to as key event relationships or KERs. The MIE identifies the initial chemical interaction with a biological target (e.g. DNA binding, protein oxidation etc.) to initiate a sequential series of biological key events (e.g. gene activation, or altered tissue development etc.) to ultimately culminate in an adverse effect relevant to risk assessment (e.g. developmental delays, disrupted reproduction, cancer, etc.)^(28,30). KEs are modular, and may not be unique to a particular adverse outcome. KERs define upstream KEs (KE_{up}) necessary for subsequent downstream KEs (KE_{down}) to occur as depicted graphically in the center box of Figure 2. For example, a change in gene expression is required for subsequent histopathological changes. Gene expression would then be defined as the KE_{up} and the histopathological changes associated with the outcome defined as KE_{down}.

Evaluating the strength of the evidence for key events in AOPs based on evolving OECD guidance⁽³⁰⁾ relies on answering and interpreting a series of questions as shown in Table I. The OECD guidance 1) prioritizes the modified Bradford Hill considerations (shown in the left-hand column of Table I); 2) identifies the defining questions to be asked relative to each Bradford Hill consideration; and, 3) provides basic criteria for evaluating confidence in the evidence based on responses to the questions⁽³⁰⁾. As the guidance is written largely in terms of single stressors (or single pathways), we adapted the questions and criteria in the context of exposures to mixtures of metals. For example, the OECD guidance identifies “biological plausibility” (number 1 in Table I) of the hypothesized key events as the most important consideration for evaluation. The key question for that consideration as written in the original OECD guidance is “a) Is there a mechanistic (i.e., structural or functional) relationship between KE_{up} and KE_{down} consistent with established biological knowledge?” We adapted that question as: “Is there a mechanistic (i.e., structural or functional) relationship between KE_{up} and KE_{down} for the mixture consistent with established biological knowledge *for the individual compounds?*” Per OECD guidance, the three key considerations include: biological plausibility, essentiality, and empirical (e.g., documentation or derivation of quantitative relationships across key events).

Similarly, evaluating the weight of evidence (e.g., low, medium, strong) as defined in OECD guidance^(29,30); requires professional judgment and is developed for each key event using a narrative-based format, again relying on our adaptation of the OECD language and guidance. For example, “Strong” evidence in the original OECD guidance is written as: “Extensive understanding of the key event relationship(s) based on extensive previous documentation and broad acceptance (e.g., mutation leading to tumours) -Established mechanistic basis” which we adapted to read: “Extensive understanding of the key event relationship(s) based on previous documentation and broad acceptance - established mechanistic basis for the mixture: One or more (appropriately designed) studies for the

primary mixture; plus several studies of individual constituents or binary combinations if the primary mixture is three or more individual constituents.” Studies based on exposure to any of the metals individually constituted moderate support – evidence was only considered strong support for biological plausibility if it was based on one or more studies involving mixtures.

3.0 Results

3.1 Toxicological and Epidemiologic Studies

The literature search identified 7 *in vitro* and 37 *in vivo* toxicological studies as shown in Table III and 22 epidemiologic studies provided in Table III 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. Table IV provides a broad overview summarizing the toxicological and epidemiologic studies available for different binary, three-way and four-way combinations of metal mixtures.

Of the 22 epidemiologic 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, and one study evaluated interactions between a metal of interest and another toxicant. The majority of epidemiologic studies limited the interaction analyses to binary combinations of metals. Furthermore, there is little consistency in the exposure measures and the statistical approaches used to examine interactions. Despite the relative lack of consistency across studies, at least one trend seems to be present in the existing epidemiologic 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).

The three-way combination studied most frequently thus far is As-Cd-Pb, with 7 *in vivo* toxicological studies (4 including an *in vitro* component), and 4 epidemiologic studies (one focused specifically on autism as an outcome and the remaining 3 focused on non-specific developmental outcomes). Although these studies evaluated potential impacts of exposure to 3 metals with the goal of identifying interactions, these interactions were typically described either in general terms relative to the impact of the individual constituent, or as different combinations of binary interactions.

Marlowe et al.⁽⁷⁷⁾ investigated associations between concentrations of As, Cd, Pb, mercury (Hg) and aluminum (Al) 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, was found to account for a significant proportion of variance in classroom teachers' ratings. Another study, Kim et al.⁽⁸⁵⁾, evaluated only the combination of Pb and Cd without any other metals. These authors dichotomized blood Cd exposure at the median, and examined effects of blood Pb levels at low versus high Cd levels. Neurodevelopment was assessed at 6 months of age using Bayley Scales of Infant Development. They 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 mechanisms, both Pb and Cd have been shown to cause oxidative stress in astrocytes^(86,92), and to interfere with calcium signaling⁶³ and neuroendocrine controls such as thyroid hormone^(93,94). The antagonistic interaction between Pb and Cd reported may be attributable 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⁽⁴⁸⁾. Cd has been shown to stimulate ovarian progesterone biosynthesis at low levels, but to inhibit the same hormone at high levels⁽⁹⁴⁾. 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 confidence in these results would be strengthened through additional 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. However, a cross-sectional study of 10,098 adults in the general population found an association between blood Pb and urinary Cd and oxidative stress markers based on NHANES data⁽⁸⁶⁾ from the general population.

There were 12 *in vivo* toxicological studies focused on Cd-Pb, and 3 of those found antagonistic effects. In a study of adult female Charles Foster rats treated intraperitoneally, Pillai et al.⁽⁴⁶⁾ found accumulation of the metals in the pituitary and lowered the membrane fluidity across exposure groups, with the greatest effect seen from Cd exposure alone. A follow-up study⁽⁴⁷⁾ demonstrated similar results in the context of effects on the hypothalamic-pituitary axis in pre-estrus rats. Neurotransmitter release as well as levels of luteinizing and follicle stimulating hormones was significantly decreased in the Cd and Cd-Pb exposed groups, but not the Pb-exposed group. Finally, coadministration of Pb and Cd to pregnant mice resulted in Pb uptake mediated by the presence of Cd as reported by Smith et al.⁽⁴⁸⁾. Three other studies focused on potential reproductive rather than developmental effects in rats found either no effects, or additive effects⁽⁴³⁻⁴⁵⁾, and three studies focused on biogenic amines did not test for interactions, although reported changes in biogenic amine levels related to behavioral outcomes⁽³⁸⁻⁴⁰⁾. Three toxicological studies with outcomes related to mitochondrial disruptions, lipid peroxidation, and divalent metal transporter found greater than additive impacts in rats with combined Cd-Pb exposures^(21,41,42).

A series of 4 *in vivo-in vitro* toxicological studies focused specifically on As-Cd-Pb exposures and glial fibrillary acidic protein (GFAP) expression in rat astrocytes. In a study of rat pups exposed throughout gestation and lactation to a combination of As-Cd-Pb, Rai et al.⁽⁶²⁾ observed a greater than additive reduction in 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 Ca²⁺ release and reactive oxygen species (ROS) generation, resulting in astrocyte apoptosis greater in the mixture than was observed for individual exposures. A series of follow-on studies⁽⁵⁹⁻⁶¹⁾ explored the influence of As-Cd-Pb on GFAP isoforms, myelin and axon development, and the role of peroxisome

proliferator-activated receptor gamma (PPARG) downregulation of GFAP and found similar synergism across outcomes.

Results from epidemiologic studies examining Pb-As interactive effects on neurodevelopment are mixed. In a correlation analysis, Moon et al.⁽⁷⁸⁾ and Marlowe et al.⁽⁷⁷⁾ 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, leading ATSDR to interpret the results as providing strong evidence for an interaction^(22,23). 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.⁽⁸¹⁾ 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.⁽⁸⁴⁾ reportedly considered As-Pb interaction in regression models, but did not report any results, leading the reader to assume no significant interaction was observed.

Marlowe et al.⁽⁷⁶⁾ investigated associations between concentrations of As, Pb, Cd, Hg, and Al, 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. Two out of 4 *in vivo* toxicological studies observed synergistic effects of combined Pb-As exposures, including increased Pb uptake and dopamine release in the rat brain⁽⁵⁴⁾ as well as a statistically significant increase in apoptosis and oxidative stress in rat brain as compared to exposures to As or Pb individually⁽⁵⁵⁾. One study did not evaluate interactions and the remaining study found a decrease in blood As in the presence of Pb as compared to As exposure alone⁽⁵⁶⁾, although the study only evaluated Pb-As and As, not Pb.

There is evidence for an interaction between Mn and other metals. In 2009, Kim et al.⁽¹⁴⁾ 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.⁽⁸⁸⁾ 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.⁽⁷⁹⁾ 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.⁽⁸⁹⁾ 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^(96,97). At high levels, Mn affects dopaminergic and cholinergic neurotransmitters and synaptic modulation, inhibits various protein transporters and enzymes, and can increase release of nitric oxide, which is involved in cellular signal transduction^(98,99). At insufficiently low levels, Mn may also have adverse neurodevelopmental effects⁽⁹⁸⁾. Although evidence suggests an inverted U-shaped relationship between Mn and neurological function⁽¹⁰⁰⁾, Claus Henn et al.⁸⁸ 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.

Several toxicological studies suggest that coexposure to Mn with other metals increases uptake of the other metals, for example, coadministration of Pb and Mn increased brain Pb levels by a factor of three as compared to lead alone⁵⁰ and increased brain Pb in five of seven brain regions⁽⁶⁸⁾. Mining waste containing a mixture of Pb, Mn and As orally administered to rats resulted in elevated levels of brain As and Mn⁽⁷⁰⁾. Andrade et al.^{67,68} found increased levels of Pb, Mn, and As in rat brain tissue following coadministration as compared to uptake by the individual metals, and found a correlation with Delta ALA-U levels in urine as well as greater than additive behavioral deficits. The combined administration of Mn and Cd increased the brain contents of Cd as compared to individual uptake in a study of rats exposed intraperitoneally⁽⁵⁸⁾.

Mining waste containing a mixture of Pb, Mn and As decreased dopaminergic transmitter following depolarization⁽⁷⁰⁾. Similarly, Mejía et al.⁽⁵⁴⁾ observed greater than additive changes in monoaminergic neurotransmitter levels in rat brains following administration of Pb and As. Increases in serotonin were observed in the cortex and midbrain, while decreases in norepinephrine were observed in the hippocampus. Leret et al.⁽⁴⁰⁾ 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. In an *in vitro* study of rat synaptosomes, Chandra et al.⁽³³⁾ found an interaction between Mn and Pb or Cd and dopamine and noradrenaline uptake.

Antonio et al.⁽³⁸⁾ observed significant inhibition of Na^+/K^+ -ATPase activity *in vivo* following coadministration of Pb and Cd, while Carfagna et al.³² noted a greater than

additive action of Pb or Cd in the presence of Mn, and Hussain et al.⁽³⁴⁾ noted the same for Pb and Mn in rat synaptosomes *in vitro*. However, Chandra et al.⁽³³⁾ in an *in vitro* study of rat synaptosomes noted only an additive interactive effect of Pb, Mn and Cd with no effect of Mn, and Lai et al.⁽³⁶⁾ noted no effects at all following combined Cd, Mn, and Al exposures in purified synaptosomes from adult male rats.

3.2 Comparative Toxicogenomics Database

Table V summarizes the results of the literature search to identify curated genes implicated across As, Pb, Mn, and Cd exposures associated with several learning disorders. These genetic targets of metal exposures provide insight into potential pathways by which *in vivo* effects have been observed, recognizing that mixtures of metals would not necessarily act on the same pathways in the same ways to result in greater than additive effects. Nonetheless, common genetic targets and impacts provide evidence that combined exposures could result in synergistic activation or deactivation of pathways relevant to expected neurodevelopmental physiological functions and may be relevant in an AOP. A minus indicates deactivation of the gene or protein regulated by the gene or a decrease in mRNA; a plus sign indicates activation of the gene or protein or an increase in mRNA.

The genes that consistently emerge across all metals and are associated with neurodevelopmental outcomes include:

BDNF—The BDNF gene regulates brain-derived neurotrophic factor, essential to the growth, maturation (differentiation), and maintenance of neuronal cells. The BDNF protein is primarily active at the synaptic junctions and is required for cellular communication and signaling and with a direct influence on synaptic plasticity. The effect of BDNF on learning and memory is associated with NMDA and non-NMDA receptor functions as well as the expression of synaptic proteins required for exocytosis⁽¹⁰¹⁾. Exposure to As, Pb, and Cd down-regulates BDNF, while exposure to Mn is associated with upregulation of this gene. A recent study documents the direct effect of Pb on BDNF⁽¹⁰²⁾.

CASP3—This gene encodes a protein, which is a member of the cysteine-aspartic acid protease (caspase) family that plays a central role in the execution-phase of neuronal cell apoptosis⁽¹⁰³⁾. It is the predominant caspase involved in cleavage of a protein associated with neuronal death in Alzheimer's disease and is related to numerous neurotoxic pathways^(104,105). This gene is upregulated across all four metals and is in the top ten interacting genes for both Mn and Cd.

CAT—This gene encodes catalase, a heme enzyme critical to defending against oxidative stress. Polymorphisms in this gene have been associated with decreases in catalase activity. Exposure to As, Cd, Mn, and Pb has been shown to down-regulate this gene, and it is one of the top ten interacting genes for As and Cd.

HMOX1—Heme oxygenase, an essential enzyme in heme catabolism, cleaves heme to form biliverdin, which is subsequently converted to bilirubin and carbon monoxide, a putative neurotransmitter. Studies have shown expression of this gene by astrocytes correlated with

lower cognitive scores in patients with sporadic Alzheimer's Disease and mild cognitive impairment⁽¹⁰⁶⁾.

IL1B—One of several pro-inflammatory cytokines in the brain related to neural plasticity at the synaptic level. Involved in synaptic function including long-term potentiation (LTP), long-term depression (LTD) and synaptic vesicle trafficking.

MAPK1/MAPK3—Mitogen-activated protein (MAP) kinases, also known as extracellular signal-regulated kinases (ERKs), act as integration points for multiple biochemical signals. They are involved in proliferation, differentiation, transcription regulation and development and participate in signal transduction pathways that control intracellular events following activation by upstream phosphorylation⁽¹⁰⁷⁾. All 4 metals upregulate these genes, with some evidence of limited down-regulation of MAPK1 by Pb. These genes are amongst the top ten interacting genes for Mn, As, and Cd.

NOS2/NOS3—Nitric oxide derived from NOS2 contributes to neurotoxicity in mouse models as well as in a model of Parkinsons disease⁽¹⁰⁸⁾.

TH—The TH gene encodes Tyrosine hydroxylase, the primary enzyme responsible for the catecholamine synthesis using molecular oxygen and iron. This gene is down-regulated by As, Mn, and Pb and is upregulated by Cd.

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

The NHANES database provides data on urinary and blood levels of As, Pb, and Cd for the 2009-2010 sampling timeframe. No NHANES data were available for Mn, so we relied on a study from Maine that reported on measured blood levels in one-to five year olds⁽¹⁰⁹⁾. In an exploratory analysis, we compared individual levels of As, Pb, and Cd from the epidemiologic studies with NHANES data to determine the potential overlap of concentrations associated with effects and observed values in the general population.

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 while NHANES data are available for urine and blood. In order to use the hair data, we used regression relationships from the literature to relate blood or urine levels to hair levels⁽¹¹⁰⁻¹¹³⁾ and maternal blood levels to cord blood^(114,115).

Figure 3 shows these results of the comparisons between biomonitoring data for the general population and exposure levels from the epidemiologic studies that evaluated mixtures. This initial screening comparison shows that, on an individual chemical basis, exposures either fall at or exceed the 95th percentile of population exposures, indicating that the majority of the U.S. population is likely not exposed to levels corresponding to those observed in the epidemiologic studies associated with outcomes. Although the 4 toxicological studies supporting a synergistic interaction of As-Cd-Pb were designed to capture environmental exposures⁽⁶⁰⁻⁶³⁾, these were typical ground water levels as observed in India (As-0.38 mg/kg; Pb-0.22 mg/kg; Cd-0.098 mg/kg), which are higher than what would be found in the United States.

3.4 Proposed Adverse Outcome Pathway

Table VI presents a list of these key events with columns for “strong” or “moderate” evidence for combined exposures based on our adaptation of the OECD guidance⁽³⁰⁾ and a list of references supporting those conclusions. Figure 4 provides a schematic relating potential initiating events with key events, key event relationships, and the outcome of interest. Overall, the strongest evidence for potential interactions is for exposures to As-Cd-Pb as demonstrated through the series of toxicological studies involving rat astrocytes⁽⁶⁰⁻⁶³⁾, and 4 epidemiologic studies, although the epidemiologic studies only evaluated binary interactions^(9,76-78) between pairs of those metals. Both the epidemiologic and toxicological studies provide the most evidence for an interaction between the presence of Mn and either As, Pb, or Cd, related to uptake and distribution of concentrations of these metals in the brain^(32,33,58,59,67-69) and for motor activity and biogenic amines in the brain⁽⁵⁰⁻⁵²⁾. There is more limited evidence for interactions involving As-Cd-Pb, and binary combinations of As-Pb, and the evidence is strongest for downstream key events with little evidence for initiating events. Evidence is strongest for greater than additive retention in the brain for Pb and Cd in the presence of Mn, while there is suggestive evidence of an inhibitory effect between Cd and Pb. Concurrently, there is evidence for the role of genetic polymorphisms for enhancing uptake, particularly for lead⁽²⁰⁾.

Most of the toxicological studies tended to provide evidence for downstream key events both biologically plausible and essential for neurodevelopment, including apoptosis^(55,60-63); oxidative stress^(41,42,52,55,64,68,70) and neurotransmitter release^(33,38,40,47,50,54,59) with less evidence for initiating events. Each of these key events is discussed next with reference to the citations in Table VI.

Molecular Initiating Event: Activation of MEK-ERK1/2 in Astrocytes—One potential initiating event involved the combined action of As-Cd-Pb is activation of the extracellular-signal-regulated kinase (ERK1/2) leading to increased intracellular Ca^{2+} followed by apoptosis in astrocytes⁽⁶⁰⁾. Extracellular-signal-regulated kinases (ERKs) are not only important regulators of cortical development generally but are known to specifically initiate an apoptotic signal via a mitochondria-dependent pathway^(154,155). Individual exposure to As in a neuroblastoma cell line⁽¹¹⁶⁾, Cd in the immature rat hippocampus^(117,118,138), Mn in developing rat striatum⁽¹¹⁹⁾ and microglia⁽¹²⁰⁻¹²¹⁾ and Pb in the immature rat hippocampus⁽¹²²⁾ has also been shown to activate the ERK1/2 pathway.

Data from the Comparative Toxicogenomics Database shows that all four metals independently are documented to increase the expression of MAPK3, one of a set of ERKs, 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, and has been specifically implicated in the development of autism. CASP3 is one of the top ten interacting genes for both Mn and Cd. A study of mechanisms of oxidative stress and apoptosis in primary cultures of rat cerebral cortex neurons found that hydrogen peroxide and nitric oxide act synergistically to induce neuronal death through apoptosis in

which activation of p38 MAPK and caspase-3 is involved⁽¹²⁶⁾. CASP3 and MAPK1/2 are genetic targets in common across As, Mn, Cd, and Pb (with CASP3 in the top ten interacting genes for Mn and Cd).

The ERK MAP kinase signaling cascade plays critical roles in brain development, learning, memory, and cognition. Samuels et al.⁽¹⁵⁴⁾ discuss the essentiality of this signaling pathway to cortical neurogenesis, cognitive function, and successful neurodevelopment and how this key event leads to developmental syndromes in humans associated with impaired cognitive function and specific developmental disorders such as autism. Moreover, a knockout mouse model has been used to demonstrate essentiality of this pathway⁽¹⁵⁵⁾. Both biological plausibility and essentiality are considered strong for this initiating event.

Key Event: Calcium signaling—There are numerous opportunities for exposure to metals to influence synaptic connections as they are being established during brain development. Many of these involve calcium signaling, which directs structural as well as functional adaptation in neurons^(136,137) and astrocytes⁽¹²⁸⁾ to establish synaptic selectivity in the developing brain. While astrocytes have long been known to support neuronal signaling, there is increasing evidence that astrocytes detect synaptic activity and engage in reciprocal signaling with neurons, again based on variations in intracellular Ca^{2+} ^(144,145). Although there is only one study that explicitly observed release of Ca^{2+} from a mixture of As-Cd-Pb⁶⁰, there is substantial data for the individual metals, including Mn, as shown in Table VI. Moreover, the essentiality of Ca^{2+} signaling in neurodevelopment and how perturbations are associated with neurotoxicity and neurodegenerative disorders is well-established, with some key references provided under “essentiality” in Table VI.

Key Event: ROS Generation and Oxidative Stress—A number of toxicological studies have observed increased production of ROS with subsequent impairment of cellular antioxidant defense systems and damage to critical biomolecules such as lipids and proteins correlated with behavioral outcomes in rodents. Rai et al.⁽⁶³⁾ exposed astrocytes to a mixture of As-Cd-Pb as well as the individual constituents and found the mixture triggered a release of Ca^{2+} with subsequent ROS generation, resulting in apoptosis of astrocytes treated with the mixture greater than would have been predicted based on exposure to the individual metals. Zhang et al.⁽⁴²⁾ reported decreased activity of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), acetylcholinesterase (AChE) and increased activity of maleic dialdehyde (MDA) in rat pups perinatally exposed to a mixture of Cd-Pb as compared to exposures to Cd and Pb individually. They reported synergistic alterations in lipid peroxidation and ultrastructural modifications in the pup brains following exposure to the mixture.

On an individual basis, each of these metals has been shown to increase oxidative stress, ROS, and subsequent neuronal or glial apoptosis via a variety of mechanisms, many of which are not completely understood. In general, evidence for biological plausibility for this key event is strong, but in terms of evidence for a synergistic effect in this specific context, the evidence is considered moderate due to a lack of consistency across tissue types and potential mechanisms. However, evidence for essentiality of oxidative stress as a potential key event in neurodevelopmental deficits is considered strong,

Key Event: Apoptosis in Astrocytes—The strongest evidence for synergistic effects is provided based on studies in astrocytes, and astrocytes are also documented targets for individual metals⁽¹²⁴⁾. Astrocytes are a source of neurotrophic factors (Table V shows that BDNF is a genetic target across all of these constituents from the comparative toxicogenomics database and in particular, Pb has a documented direct impact on BDNF⁽¹⁰²⁾; reduced glial fibrillary acidic protein expression interferes with astrocyte production and modulates synaptic efficiency in the central nervous system^(131,132) and the As-Cd-Pb combination has been observed to act synergistically specifically on GFAP in toxicological studies with rats⁽⁶⁰⁻⁶³⁾. Astrocytes control ion homeostasis, glutamate uptake at the synapse and provide metabolic support for neighboring neurons^(143,144). Astrocyte contact is necessary for the synaptic maturation of developing neurons^(142,146,148,149), and the coadministration of As-Cd-Pb revealed a greater than additive effect on apoptosis of astrocytes in rats⁽⁶⁰⁻⁶³⁾ and As-Pb on apoptotic markers in the cerebral cortex, hippocampus and cerebellum in offspring of maternally exposed rats⁽⁵⁵⁾. Toxicological studies show that individual exposure to As, Cd, Mn, and Pb all independently have been associated with apoptosis or apoptotic markers in neuronal and glial tissues as referenced in Table VI, providing strong evidence for biological plausibility of this key event.

A recent study demonstrates that neuroglial signaling involving astrocytes differs significantly in the adult versus developing brain⁽¹⁵⁷⁾, highlighting both the potential vulnerability of perinatal exposures as well as the evolving nature of our understanding of expected neurodevelopment. Further, new evidence suggests that astrocyte synaptic regulation is not restricted to the active tripartite synapse but can be manifested through astrocyte signaling at synapses relatively distant from active synapses, a process termed lateral astrocyte synaptic regulation⁽¹⁵⁰⁾, demonstrating strong evidence for the essentiality of apoptosis as a key event.

Key Event: Neurotransmitter Release—Several toxicological studies have explored biogenic amines and neurotransmitter release in mixtures as compared to individual metals and found some evidence of synergistic action or changes relative to the mixture. For example, Mejía et al.⁽⁵⁴⁾ assessed levels of three monoamines: dopamine, norepinephrine, and serotonin (DA, NE, and 5-HT) and of two metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindole-3-acetic acid (5-HIAA), in specific brain regions following 14 days of oral administration of As, Pb, or the As-Pb mixture. They found exposure to the mixture led to a 38% decrease of NE in the hippocampus and a 100% and 90% increase of 5-HT in the midbrain and frontal cortex over control values, while there were no statistical differences from controls in the individually exposed As or Pb rats. Three studies focused on the Cd-Pb mixture^(38,40,47), but none were designed to test interaction effects from the mixture. Antonio et al.⁽³⁸⁾ and Leret et al.⁽⁴⁰⁾ observed statistically significant increases in DOPAC, 5-HT and 5-HIAA contents in the cerebellum and hippocampus in rat offspring exposed to a Cd-Pb mixture throughout pregnancy and lactation, but without any comparison to individual Cd or Pb exposure. Pillai et al.⁽⁴⁷⁾ only evaluated the hypothalamic-pituitary axis of adult rats. Chandra et al.⁽⁵⁰⁾ exposed adult rats to Mn, Pb, or a Mn-Pb mixture and observed dose-related decreases in the levels of DA, NE and 5-HT in brain tissue and concomitant behavioral outcomes.

Evidence for biological plausibility for this key event is strong in a general sense given the evidence from studies with individual metals and some combinations, but the lack of concordance across tissue types and life stages for the studies in mixtures provides only moderate evidence of potential interaction. Essentiality for this key event is considered strong based on a putative understanding of the role neurotransmitter release plays in learning and development^(101,136).

Adverse Outcome: Learning and Cognitive Deficits—Three epidemiologic studies explored exposure to mixtures of As-Cd-Pb⁽⁷⁶⁻⁷⁸⁾ although only evaluated binary combinations with respect to interaction and found interactions between As-Pb and Cd-Pb. This is the same mixture from the series of toxicological studies reporting a greater than additive effect of the mixture on apoptosis in astrocytes via increases in Ca^{2+} and ROS initiated by ERK1/2⁽⁶⁰⁻⁶³⁾, providing moderate to strong evidence of a complete pathway from initiating event to adverse outcome.

Both Claus Henn et al.⁽⁸⁸⁾ and Kim et al.⁽¹⁴⁾ found an interaction between high levels of Mn and Pb exposures and performance on neurodevelopmental tests in epidemiologic studies of children. Evidence from the toxicological studies is equivocal with respect to key events, although the evidence is stronger for observed increases in brain tissue concentrations of Pb in the presence of Mn⁽⁴⁹⁻⁵¹⁾.

Initiating Events for Individual Metals—There are a number of molecular initiating events one could hypothesize based on data for individual metals and known neurodevelopmental pathways, particularly those that lead to known downstream events. For example, acetylcholinesterase binding is known to inhibit acetylcholinesterase resulting in oxidative stress, and there is data for individual metals for this potential MIE but not for combinations of metals. As there are no studies of these metals as mixtures and therefore no way to determine at this time whether the combined effect on converging key events could be greater (or less) than additive, these potential MIEs are left unspecified.

Empirical Support for Key Event Relationships—Data are not sufficient at this time to develop quantitative estimates of relationships between key events. However, as shown in Figure 3, a preliminary comparison of biomarkers of exposure in the general population and exposures as determined from the epidemiologic studies suggests that the majority of the U.S. population is likely not exposed at levels at which effects have been observed.

4.0 Discussion

As the magnitude of individual epidemiologic and toxicological studies grows, systematic review offers a structured approach for considering specific research or public health questions, and there is great interest in developing guidelines and best practices⁽¹⁵⁸⁾, particularly for identifying and evaluating study quality and reliability⁽¹⁵⁹⁾ and for combining qualitative and quantitative data⁽¹⁶⁰⁾. In the context of environmental exposures, and as risk assessment methodology evolves to capture growing evidence on the ways in which environment, genes, and exposures combine to lead to adverse outcomes, the AOP construct is emerging as an integrative framework for evaluating the evidence supporting or

refuting key events across the continuum from molecular initiating events to adverse outcomes in the context of expected physiological function^(161,162). The AOP framework extends the results of a systematic review by providing a higher level depiction and mechanistic understanding of the sequence of key toxicological events culminating in an adverse outcome⁽¹⁶³⁾. Key events are essential, although not necessarily sufficient, measurable changes in biological states in the progression from a defined biological perturbation to a specific adverse outcome⁽¹⁶²⁾.

In the context of neurotoxicity, neurodevelopmental pathways in the developing human brain are not fully understood⁽¹⁶⁴⁻¹⁶⁵⁾, precluding the development of definitive AOPs. However, much is known about individual key events. A workshop organized under the auspices of the European Union Reference Laboratory for Alternatives to Animal Testing resulted in ten proposed, although not fully developed, putative AOPs⁽¹⁶³⁾ focused on individual chemicals. Developed independently, the results presented here include many of the key events identified through the workshop⁽¹⁶³⁾.

Most examples of AOPs currently focus on individual chemical exposures evaluated in the context of a single pathway from MIE to adverse outcome. There is strong evidence that the key events shown in Figure 4 are both biologically plausible contributors to non-specific learning and developmental deficits, and essential to neurodevelopment, although defining the quantitative relationships between key events still requires further analysis. Exposure to multiple chemicals, as well as non-chemical stressors, will undoubtedly lead to interactions that may result in different mechanisms of toxicity than observed for individual constituents, as well as leading to differences in outcomes (e.g., synergistic, antagonistic) than would be predicted for individual chemicals. A key challenge in developing AOPs is that causation is induced logically, not observed empirically⁽¹⁶⁶⁾, which is complicated enough when considering one chemical-one outcome, let alone the assessment of mixtures and interactions. Ultimately, AOPs will likely be developed in terms of networks to capture these potential overlaps and feedbacks. Bayesian networks, agent-based modeling, and other computational frameworks with common interfaces will be required to support quantitative descriptions of pathways⁽¹⁶¹⁾. There are many challenges and complexities associated with AOP development, but also many efforts to identify and describe best practices going forward^(161-163,186-187).

Risk assessment requires quantitative estimates of toxicity, which requires additional modeling⁽¹⁶⁷⁻¹⁷²⁾. The evidence suggests that exposure to Mn in the presence of other metals increases uptake into the brain, which impacts the absorbed dose. Physiologically-based pharmacokinetic (PBPK) modeling approaches can be used to inform a better understanding of the relationship between exposed and absorbed dose and target organs, but typically these are developed for individual compounds. For example, PBPK models have been developed for Mn^(173,174), As^(175,176), Cd⁽¹⁷⁷⁻¹⁷⁹⁾, and Pb (the Integrated Exposure UptakeBiokinetic [IEUBK] Model for Lead in Children, available from www.epa.gov/superfund/lead/products.htm). There are few published studies that extend the analysis to mixtures of metals, although these kinds of PBPK approaches are being developed for other kinds of mixtures^(180,182) and for predicting *in vivo* exposures associated with *in vitro* assay results⁽¹⁸²⁻¹⁸⁴⁾, including the specific context of neurodevelopmental effects and

neuroprogenitor proliferation⁽¹⁸⁵⁾. For “traditional” epidemiologic and toxicological studies, complex statistical methods are being developed for estimating the effects of exposure to mixtures, including supervised clustering methods (e.g., tree-based methods, random forests), Bayesian approaches, dimension reduction approaches (e.g., principal component analysis), and weighted quartile score regression strategies^(1,167-172). These methods also consider potential thresholds, which may be relevant both for individual chemicals (e.g., there is a requirement for some level of Mn as an essential nutrient) as well as mixtures (e.g., physiological pathways and events may involve initiation thresholds). In the context of AOP development informing risk assessment, human dosimetry methods applied to effect levels based on non-testing approaches and *in vitro* data can be used to compare to exposure levels or develop regulatory values. For example, Patlewicz et al.⁽¹⁸⁷⁾ discuss the use of exposure:activity ratios (EAR) to interpret biological activity based on AOPs for skin sensitization, endocrine disruption, and liver tumor promotion in rodents.

This review provides evidence of synergistic effects related to exposures to mixtures of several metals, notably As-Cd-Pb and Mn in the presence of As, Cd, or Pb, and provides a foundational example for the evaluation of key events and outcomes relevant both to this specific mixture as well as more broadly applicable to other neurotoxicants based on an application of the evolving OECD guidance for developing AOPs. We propose a series of key events in keeping with related efforts to develop putative AOPs for neurodevelopmental pathways with the goal of ultimately estimating quantitative linkages for key event relationships to better inform regulatory decision making in the face of scientific complexity.

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Figure 1.
Schematic Showing the Intersection of Domains of Evidence

Figure 2.
General Schematic for Adverse Outcome Pathway Development

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

Figure 4.
Proposed Pathways of Potential Neurotoxicity of As-Cd-Mn-Pb

Guidance for Assessing the Relative Level of Confidence in Evaluating Pathways for Adverse Outcome Pathway Development (Adapted from Ref 30)

Table 1

1. Support for Biological Plausibility ¹	Defining Question	High, Strong ^{2,3}	Moderate	Low, Weak
	Is there a mechanistic (i.e., structural or functional) relationship between KE _{up} and KE _{down} for the mixture consistent with established biological knowledge for the individual compounds?	Extensive understanding of the KER based on previous documentation and broad acceptance - established mechanistic basis for the mixture. One or more (appropriately designed) studies for the primary mixture; plus several studies of individual constituents or binary combinations if the primary mixture is three or more individual constituents.	The KER is plausible based on analogy to accepted biological relationships of individual compounds but scientific understanding is not completely established in the mixture. Similar pathway but observed only for individual constituents.	There is empirical support for a statistical association between KEs, but the structural or functional relationship between them is not understood. Studies for only some of the individual constituents.
MIE => KE1	Biological plausibility is (high - medium - low) and rationale. Rationale includes: observed dose-response, concordance or conflicting evidence across studies, temporality (exposure precedes effect); individual contaminant mode of action consistent with that observed for mixture.			
KE1 => KE2				
KE2 => KE3				
2. Support for Essentiality of KEs	Defining Question	High, Strong	Moderate	Low, Weak
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs (e.g., stop/reversibility studies, antagonism, knock out models, etc.)	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE (e.g., augmentation of proliferative response (KE _{up}) leading to an increase in KE _{down} or adverse outcome)	No or contradictory experimental evidence of the essentiality of any of the KEs
MIE	Essentiality of the MIE is (high - medium - low) and rationale. Rationale is based on experimental evidence and the concordance across studies.			
KE1				
KE2				
3. Empirical Support for KERs	Defining Question	High, Strong	Moderate	Low, Weak
	Does the empirical evidence support that a change in KE _{up} leads to an appropriate change in KE _{down} ? Does KE _{up} occur at lower doses and earlier time points than KE _{down} and is the incidence of KE _{up} > than that for KE _{down} ? Are there inconsistencies in empirical support across taxa, species, and stressors that don't align with expected pattern for the hypothesized AOP?	Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. Extensive evidence for temporal, dose response, and incidence concordance and no or few critical data gaps or conflicting data.	Demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all); and/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesized AOP.
MIE => KE1 KE1 => KE2 KE2 => KE3	Empirical support of the MIE => KE1 is (low - medium - high) and rationale. Strong support is demonstrated by 1) change in KE _{down} can be precisely predicted based on a relevant measure of KE _{up} ; 2. Uncertainty in the quantitative prediction can be precisely estimated from the variability in the relevant measure of KE _{up} ; 3. Known modulating factors are accounted for in the quantitative description; and, 4. There is evidence that the quantitative relationship between the KEs generalizes across the relevant applicability domain of the AOP.			

Notes:

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KE = key event (up preceding or leading to down)
 KER = key event relationship(s) between upstream and downstream events
 MIE = molecular initiating event
 AO = adverse outcome

¹Rank ordered Bradford Hill considerations presented in OECD (30) based on their interpretation of Ref 29.

²The guidance for “high”, “moderate” and “low” is based on the Users Handbook Supplement to OECD 2013. As more AOPs are developed, this guidance will likely continue to be refined.

³“Direct evidence” implies experiments designed to specifically evaluate that KE. “Indirect evidence” relates to inference from empirical studies.

Toxicological Studies Evaluating One or More Metals

Table II

Reference	Ref	Target Organ	Exposure	N	Response	Methods	Conclusions	Range of Concentrations	Evidence for Interaction
<i>in vitro</i> studies									
Ben Fredj et al. 2010	31	Chinese hamster ovary cells transfected with heat-shock protein 47 promoter	Ni, Cd, Pb	NA	Stress response	Ternary diagram	Highest stress response effect observed at high Ni and Cd for several Pb levels	Unknown	+ at higher concentrations
Carfagna et al. 1996	32	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	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	33	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	Pb/Cd inhibition of Na ⁺ /K ⁺ -ATPase additive; Mn no effect. Interaction of Mn with Pb or Cd produced inhibition on the uptake of DA and noradrenaline	0 - 100 µM	+, no effect
Hussain et al. 1987	34	Pretreated rat striatal synaptosomes	Pb ²⁺ , Mn ²⁺	30	Monoamine uptake and Na ⁺ and K ⁺ -ATPase	ANOVA	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	35	Brain protein from rats	Pb, Mn	NA	protein binding		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	36	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	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	Not possible to determine degree of interaction
Lai et al. 1981	37	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	Individual inhibitory effects on DA uptake by forebrain synaptosomes with differential interaction	0.1 - 100 µM	Not possible to determine degree of interaction
<i>in vivo</i> studies									

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Reference	Ref	Target Organ	Exposure	N	Response	Methods	Conclusions	Range of Concentrations	Evidence for Interaction
Antonio et al. 2002	38	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	Reduced hemoglobin, hematocrit; Na ⁺ /K ⁺ -ATPase significantly reduced (normal following removal of exposure); reductions in motor activity; increased DA and 5-HT	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
Nation et al. 1990	39	Adult male Sprague-Dawley rats exposed via diet for 60 days	Pb, Cd	32	Behavioral and motor outcomes	Behavior, ANOVA	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
Leret et al. 2003	40	Pregnant Wistar rats exposed via drinking water	Cd acetate; Pb acetate	24 pups	Behavior, biogenic amines	ANOVA	Increases in anxiety-like behavior; increased hippocampal DA and 5-HT	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
Bizarro et al. 2003	41	Inhalation of CD-1 mice 1 hour twice a week for four weeks	Pb, Cd	72	Mitochondrial ultrastructure of Sertoli cells	ANOVA	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	+
Gu et al. 2008	21	Intragastrical perfusion of Sprague-Dawley rats once a day for six weeks	Pb, Cd	24	Divalent metal transporter 1 (DMT1)	ANOVA, t-test	Greater than additive protein levels of DMT1 from mixture	Pb 20 mg/kg-d, Cd 2 mg/kg-d and combined	+
Zhang et al. 2009	42	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	Greater than additive effect on mitochondrial swelling and other ultrastructural modifications; decrease in glutathione peroxidase, superoxide dismutase, catalase, acetylcholinesterase; increase in maleic dialdehyde	Pb (300 mg/l); Cd (10 mg/L) and combined	+
Nampoothiri and Gupta 2008	43	Charles female rats treated subcutaneously	Pb, Cd	4 groups of 12-13 rats	Biochemical effects on reproductive performance, placenta, and ovary	ANOVA	Pb and Cd alone or in combination had no significant effect on reproductive performance	0.05 mg/kg-d individually and combined	no effect
Pillai et al. 2009	44	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	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 individually, 0.025 mg/kg-d combined)	no effect
Pillai et al. 2010	45	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	No significant effect on reproductive performance; key steroidogenic enzymes impacted but not synergistically	lead acetate, cadmium acetate (0.05 mg/kg-d individually, 0.025 mg/kg-d combined)	no effect

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Reference	Ref	Target Organ	Exposure	N	Response	Methods	Conclusions	Range of Concentrations	Evidence for Interaction
Pillai et al. 2002	46	Adult female Charles Foster rats treated intraperitoneally	Pb, Cd	unknown	Pituitary membrane, N^+/K^+ -ATPase	ANOVA	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	47	Adult female Charles Foster rats treated intraperitoneally	Pb, Cd	unknown	Hypothalamic-pituitary axis	ANOVA	Decrease in 5-HT and NE 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	–
Smith et al. 2012	48	Pb and Cd administered to Ballb/c mice in feed for 18 days	Pb, Cd	9	Contaminant concentrations in coexposed pregnant and nonpregnant mice	ANOVA	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	–
Betharia and Maher 2012	49	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	Females but not males exposed to Pb or Mn displayed behavioral deficits; no effect of mixture observed. Pharmacokinetic interaction	Pb (10 Mg/ml); Mn 2 mg/ml	no effect
Chandra et al. 1981	50	Male Albino rats exposed to Mn in drinking water and intraperitoneally to lead acetate	Pb²⁺, Mn²⁺	8 groups of 15 each	Changes in motor activity and biogenic amines in the brain	t-test	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	51	Rats exposed intraperitoneally on day 0 of gestation	Pb, Mn	40 female, 10 male	Brain growth and biochemicals in pups	t-test	Pb accumulation in the brain significantly increased 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	+
Malhotra et al. 1984	52	Fe-deficient male Albino rats exposed intraperitoneally (Pb) and drinking water (Mn)	Pb²⁺, Mn²⁺	80	Lipid peroxide formation; contents of metals in the brain	ANOVA	Concurrent exposure to Pb and Mn 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.)	+
Fairhall and Miller 1941	53	White rats exposed orally	Pb, As	177	Mortality, weight loss, food refusal, diarrhea, poor posture, gait, tissue distribution	NA	Calcium arsenate > lead arsenate > 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
Mejía et al. 1997	54	Male BALB/c mice exposed via gastric intubation over 14 days	Pb, As	NA	Levels of DA NE 5-HT and metabolites	ANOVA, Tukey's HSD, t-test	Mixture showed higher accumulation of Pb and lower As than individually; mixture	Pb (74.0 mg/kg-d), As (8.0 mg/kg-d)	+

Reference	Ref	Target Organ	Exposure	N	Response	Methods	Conclusions	Range of Concentrations	Evidence for Interaction
							decreased NE in hippocampus and increased 5-HT in cortex and midbrain		
Sannadi et al. 2013	55	Wistar rats exposed via drinking water from GD6 to PND21	Pb acetate, As	24 dams; number of pups unknown	Oxidative stress, apoptosis throughout brain	ANOVA, student Newman Kuhl's post-hoc	Statistically significant increase in oxidative stress (caspase-3), apoptosis, accumulation in cerebral cortex	Pb (0.2%); As (50 mg/kg); Pb and As together	+
Diacomanolis et al. 2013	56	8-wk old Sprague-Dawley rats via one-time gavage	Pb, As	8 groups of 5 each	Bioavailability and pharmacokinetics	Leven's, Tukeys, Dunnett's, Bonferroni	Decrease in As blood with increasing Pb exposure; decreased absorption of As in the presence of Pb	As (0.5, 5, 15) mg/kg; As (2.5 mg/kg) + Pb (0.5, 10, 20 mg/kg))	–
Hochadel and Waalkes 1997	57	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	As pre-treatment protected against Cd-induced lethality; Cd pretreatment did not alter As toxicity	22.5 µM NaAsO ₂ /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
Lal et al. 1980	58	Male Albino rats exposed intraperitoneally for 30 days; pairwise exposure (Mn + one other)	Cd²⁺, Mn²⁺, Zn²⁺, Hg₂²⁺	8 groups of 10 each	Tissue distribution	t-test	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 ²⁺ (0.5 mg/kg)	+
Shukla and Chandra 1982	59	Male Albino rats exposed intraperitoneally for 30 days	Cd²⁺, Mn²⁺, Zn²⁺, Hg₂²⁺	8 groups of 20 animals	Biogenic amines	NA	Increased DA and NE; decrease in 5-hydroxyindoleacetic acid relative to individual	Mn ²⁺ (4 mg/kg), Zn ²⁺ (1.0 mg/kg), Cd ²⁺ (0.5 mg/kg), Hg ₂ ²⁺ (0.5 mg/kg)	+, no effect
Rai et al. 2014	60	Gavage to pregnant and lactating Wistar rats and postweaning pups up to 2 months	As, Cd, Pb	30 animals in 6 groups	Astrocytes from cerebral cortex and cerebellum of developing rats	ANOVA, Student-Newman-Keuls; t-test	Greater than additive downregulation in viability and increased apoptosis of astrocytes, induced by ERK/JNK signaling, exploration of PPARG in downregulating GFAP	Typical ground water concentrations: As-0.38 mg/kg; Pb-0.22 mg/kg; Cd-0.098 mg/kg	+
Rai et al. 2013	61	Gavage to pregnant and lactating Wistar rats and postweaning pups up to 2 months	As, Cd, Pb	30 animals in 6 groups	Astrocytes from cerebral cortex of adult rats	ANOVA, Student-Newman-Keuls; t-test	Mixture influences composition of GFAP isoforms, suppressed GFAP promotes apoptosis in astrocytes	Typical ground water concentrations: As-0.38 mg/kg; Pb-0.22 mg/kg; Cd-0.098 mg/kg	+
Rai et al. 2013	62	Gavage to pregnant and lactating Wistar rats and postweaning pups up to 2 months	As, Cd, Pb	30 animals in 6 groups	Myelinating white matter in cerebral cortex	Combination index	Dose-dependent, synergistic hypomyelination in cortex; reduced axon area and myelin density; apoptosis in oligodendrocytes, astrocytes	Typical ground water concentrations: As-0.38 mg/kg; Pb-0.22 mg/kg; Cd-0.098 mg/kg	+
Rai et al. 2010	63	Gavage to pregnant and lactating Wistar rats and postweaning pups up to 2 months	As, Cd, Pb	30 animals in 9 groups	Astrocytes from cerebral cortex and cerebellum of developing rats	ANOVA, Student-Newman	Greater than additive downregulation in viability and increased apoptosis of astrocytes, induced by	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	+

Reference	Ref	Target Organ	Exposure	N	Response	Methods	Conclusions	Range of Concentrations	Evidence for Interaction
							ERK/JNK signaling, increase in Ca^{2+} and ROS generation	mg/kg; individual groups Pb 2.22, 6.66 mg/kg, Cd, 0.98, 2.94 mg/kg, As 3.8, 11.4 mg/kg	
Fowler et al. 2004	64	Sprague-Dawley rats exposed to drinking water at 30, 90, 180 days	As, Cd, Pb	168	Molecular parameters of oxidative stress in renal and hematopoietic systems	Factorial design	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)	+
Mahaffey and Fowler 1977	65	Diet exposed male Sprague-Dawley rats	As, Cd, Pb	168	Blood chemistry, tissue distribution	Factorial design	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	66	Diet exposed male Sprague-Dawley rats	As, Cd, Pb	168	Blood chemistry, tissue distribution	Factorial design	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
Andrade et al. 2014	67	Male Wistar rats exposed to As via drinking water for 8 days; Pb and Mn via intraperitoneal injection (8 doses)	As, Mn, Pb	6 rats in 5 groups	Tissue distribution		2.5 times higher Pb in brain tissue for the mixture;	Pb (5 mg/kg), As (60 mg/L), Mn (10 mg/kg) individual and in combination	+
Andrade et al. 2013	68	Male Wistar rats exposed to As via drinking water for 8 days; Pb and Mn via intraperitoneal injection (8 doses)	As, Mn, Pb	6 rats in 5 groups	Open-field spontaneous motor activity; urinary delta-ALA; uptake into brain tissue	Mann-Whitney; spearman's; ROC curves	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	+
Shukla and Chandra 1987	69	Pb in drinking water with intraperitoneal Mn or Cd administered to male Albino rats for 30 days	Cd, Mn, Pb	300	Contaminant distribution in the bodies of growing rats	ANOVA Duncan's Multiple range test	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 <i>ad libitum</i> , 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 <i>ad libitum</i> with Mn^{2+} 1 mg/kg, Pb^{2+} 5 mg/l <i>ad libitum</i> with Mn^{2+} 4 mg/kg, Pb^{2+} 5 mg/l <i>ad libitum</i> with Cd^{2+} 0.1 mg/kg, Pb^{2+} 5 mg/l <i>ad libitum</i> with Cd^{2+} 0.4 mg/kg	+
Rodriguez et al. 1998	70	Dopamine release from 60 adult male Wistar rats orally and subchronically exposed to mining waste	As, Cd, Mn, Pb	60	Basal dopamine release under long-standing depolarization through high-potassium perfusion	<i>in vivo</i> microdialysis ANOVA, Tukeys HSD	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
Eisenhans et al. 1987	71	Sprague-Dawley rats exposed to binary combinations via the diet	As, Cd, Mn, Pb, Cu, Fe, Ni, Zn	28	distribution in the body	Spearman correlation coefficient	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	+, –

Reference	Ref	Target Organ	Exposure	N	Response	Methods	Conclusions	Range of Concentrations	Evidence for Interaction
Jadhav et al. 2007	72	Male Wistar rats exposed via drinking water for 30, 60, 90 days	As, Cd, Mn, Pb, Cr, Fe, Hg, Ni	5 groups of 10 each	oxidative stress in erythrocytes	ANOVA, Duncan's <i>post-hoc</i>	Effects observed only at highest doses; no effects at 1×, 10×	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
Chandra et al. 1980	73	Male ITRC mice treated intraperitoneally for 40 days	Cu, Fe, Mn	120 divided into 6 groups	ADME; Metal absorption	atomic absorption spectrophotometer	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)	+, –

DA - dopamine
NE - norepinephrine
5-HT - serotonin
RBC - red blood cells

Table III

Epidemiologic Studies Involving One or Metals

Reference	Ref	Study Design	N	Metals Evaluated	Exposure Measure	Exposure Levels	Outcome	Interactions between toxicants assessed?	Conclusions
Geier et al. 2012	74	cross-sectional	18 children age 1-6 yrs	As, Cd, Pb, Mn, Hg, Cr, Co, Ni, Al, U, Sn	Hair	$\mu\text{g/g}$: 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), 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 $\pm$ 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)	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.
Haynes et al. 2011	75	ecological	88 Ohio counties	As, Cd, Pb, Mn, 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	Youth adjudications for criminal activity	No	Airborne exposure to manganese, mercury, and particulate matter correlated with increased risk of adjudication.
Adams et al. 2009	9	cross-sectional	63 children age 3-8 yrs	As, Cd, Pb, Sb, Sn, Ti, Hg, Wb, Al, Sn	Urine (metals measured before and after chelation with DMSA)	Means: Pb: 1.3 $\mu\text{g/g}$ creatinine; As: 32 $\mu\text{g/g}$ creatinine; Cd: 0.38 $\mu\text{g/g}$ creatinine	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.
Marlowe et al. 1985a	76	cross-sectional	80 elementary children	As, Cd, Pb, Hg, Al	Hair	Pb 6.65 ppm, As 2.94 ppm, Hg 0.99 ppm, Cd 0.64 ppm, Al 9.41 ppm	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	77	cross-sectional	69 elementary children	As, Cd, Pb, Hg, Al	Hair	Pb 6.65 ppm, As 2.94 ppm, Hg 0.99 ppm, Cd 0.64 ppm, Al 9.41 ppm	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	78	cross-sectional	69 elementary children	As, Cd, Pb, Hg, Al	Hair	Pb 6.65 ppm, As 2.94 ppm, Hg 0.99 ppm, Cd 0.64 ppm, Al 9.41 ppm	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

Reference	Ref	Study Design	N	Metals Evaluated	Exposure Measure	Exposure Levels	Outcome	Interactions between toxicants assessed?	Conclusions
Lin et al. 2013	79	prospective cohort	230 mother-infant pairs	As, Pb, Mn, Hg	Cord blood	Mn= 47.90 µg/L (range, 17.88–106.85 µg/L), Pb=11.41 µg/L (range 0.16–43.22 Mg/L), As=4.05 Mg/L (range, 1.50–12.88 Mg/L) and Hg=12.17 Mg/L (range, 1.53–64.87 Mg/L)	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
McDermott et al. 2011	80	retrospective cohort	3988 infants	As, Pb, Mn, Ba, Cr, Cu, Hg, Ni	Soil at maternal residence during pregnancy	Bayesian kriging	Intellectual disability as documented by school placement or disability service	Yes (between As, Pb)	The probability of intellectual disability was significantly associated with the interaction between Pb and As for normal weight for gestational age infants
Calderon et al. 2001	81	cross-sectional	41 high exposure; 39 low exposure	As, Pb	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	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.
Obrenovich et al. 2011	82	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	ASD cases compared to controls on 1) erythrocyte transketolase, 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	83	hospital-based birth cohort	100 mother-infant pairs in Nepal	As, Pb, Zn	Cord blood	Pb 6.83–220.8 µg/L, As 0.51–9.58 µg/L, Zn 1299–6430 µg/L	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.
Rosado et al. 2007	84	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/	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 (no results shown)	Urinary As associated with poor performance on WISC, independent of Pb
Kim et al. 2013	85	prospective longitudinal study	884 women-infant pairs	Cd, Pb	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;	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

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Reference	Ref	Study Design	N	Metals Evaluated	Exposure Measure	Exposure Levels	Outcome	Interactions between toxicants assessed?	Conclusions
						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			MDI and PDI were only significant at high Cd levels
Lee et al. 2006	86	cross-sectional	10,098 adult participants	Cd, Pb	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.	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
Thatcher et al. 1982	87	cross-sectional	149 children 5-16 yr	Cd, Pb	Hair	5 - 22 ppm Pb, 0.5 - 1.3 Cd	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 Cd has a significantly stronger effect on verbal IQ than does Pb and that Pb has a stronger effect on performance IQ than does Cd
Claus Henn et al. 2012	88	prospective longitudinal study	450 Mexican children	Pb, Mn	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	Bayley Scales of Infant Development-II	Yes	Observed evidence of synergism between Pb and Mn whereby Pb toxicity was increased among children with high Mn coexposure.
Kim et al. 2009	14	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)	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
Lucchini et al. 2012	89	cross-sectional	299 adolescents 11-14 yrs	Pb, Mn	Blood Pb, Blood/Hair/Air/Soil Mn	(BPh) 1.71 Mg/dL (median 1.5, range 0.44–10.2), (BMn) 11.1 µg/dL (median 10.9 range 4.00–24.1)	Wechsler Intelligence Scale for Children (WISC) and the Conners-Wells' Adolescent Self-Report Scale Long Form (CASS:L)	Yes	No interaction. Main effects of Pb: 2.4 IQ pt reduction for a two-fold increase of blood Pb. No main effect of Mn
Roberts et al. 2013	90	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	Autism Spectrum Disorder	Evaluated overall measure of metal exposure	Perinatal exposures to the highest versus lowest quintile of diesel, Pb, Mn, Hg, 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 Hg)

Reference	Ref	Study Design	N	Metals Evaluated	Exposure Measure	Exposure Levels	Outcome	Interactions between toxicants assessed?	Conclusions
Khan et al. 2011	91	cross-sectional	201 Bangladeshi Children 8–11 years of age	As, Mn	Tube well water	As (µg/L) 0.9–18.0, Mn (µg/L) 6.3–33.9, Pb (µg/L) 36.7–245.7	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.
Wasserman et al. 2011	13	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	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	15	cross-sectional	32 children 11-13 yrs	As, Cd, Mn	Hair	Mn 471.5 ppb, As 17.8 ppb, Cd 57.7 ppb	Wechsler Abbreviated Intelligence Scale (WASI), Wide Range Assessment of Visual Motor Ability (WRAYMA), 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 IV

Summary of Toxicological and Epidemiologic Studies and Metal Interactions

Interaction	EPIDEMIOLOGIC			TOXICOLOGICAL			Evidence for Interaction
	Outcome	Number of Studies	Evidence for Interaction	Outcome	Number of Studies	Evidence for Interaction	
As, Cd, Pb, Mn	Autism	1 [74]	Did not test	NA	NA	NA	NA
As, Cd, Pb, Mn	Behavior	1 [75]	Did not test	Basal dopamine release, oxidative stress in erythrocytes	3 <i>in vivo</i> [70-72]	Synergism at highest dose for oxidative stress in 1 study [71]	
As, Cd, Pb	Autism	1 [9]	Did not test	NA	NA	NA	NA
As, Cd, Pb	Behavior, Cognition	3 [76-78]	Observed interactions in binary combinations of As-Pb, Cd-Pb	Tissue distribution, astrocytes	7 <i>in vivo</i> [60-66]	4 <i>in vivo</i> studies reported synergism on a) apoptosis of astrocytes [60-63], b) oxidative stress [64]; no effect on tissue distribution [65,66]	
As, Cd, Mn	Cognition	1 [15]	Observed a binary interaction between As-Mn	NA	NA	NA	NA
As, Pb, Mn	Cognition	2 [79,80]	1 study observed an interaction between As-Pb [79], the other Pb-Mn [78]	Tissue distribution; behavioral outcomes	2 [67,68]	Increased levels of all 3 metals in rat brain tissue as compared to individual uptake; greater than additive behavioral deficits	
Cd, Pb, Mn	NA	NA	NA	Uptake	2 <i>in vitro</i> , 1 <i>in vivo</i> [32,33,69]	Increased uptake across all three metals [69]	
As, Pb	Cognition	4 [81-84]	[81,84] Reported qualitative interaction	Tissue distribution; neurotransmitter release	4 <i>in vivo</i> [53-56]	Increased Pb uptake in brain (decreased As); synergistic neurotransmitter release [54]; decrease in blood As [55]; synergistic oxidative stress/apoptosis [56]	
As, Mn	Cognition	2 [13,91]	No interaction in cross-sectional studies	NA	NA	NA	NA
As, Cd	NA	NA	NA	Mortality	1 <i>in vivo</i> [57]	As pre-treatment protected against Cd-induced lethality; Cd pretreatment did not alter As toxicity	
Cd, Pb	Cognition	3 [85-87]	1 antagonistic [84]; 2 did not test [85-86]	Uptake, neurotransmitter release, mitochondria	12 <i>in vivo</i> [21,38-48]	Antagonism in uptake [48]; Pb mediated Cd effect in pituitary [46,47]; synergism in mitochondrial swelling, lipid peroxidation [21,41,42]	
Cd, Mn	NA	NA	NA	Mono/biogenic amine uptake	2 <i>in vitro</i> , 2 <i>in vivo</i> [36,37,58,59]	Presence of Mn increased uptake of Cd in the brain [57,58]	
Pb, Mn	Cognition	4 [14,88-90]	2 synergism (prospective longitudinal; cross-sectional)	Motor activity, biogenic amines	2 <i>in vitro</i> , 4 <i>in vivo</i> [34,35,49-52]	3 <i>in vivo</i> studies reported synergism [50-52]; 1 reported no effect [49]; 2	

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EPIDEMIOLOGIC			TOXICOLOGICAL			
Interaction	Outcome	Number of Studies	Evidence for Interaction	Outcome	Number of Studies	Evidence for Interaction
			[14,87], 1 no interaction [88], 1 didn't test [89]			<i>in vitro</i> studies reported synergism [34,35]

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

Gene	Pathway Description (obtained and adapted from the National Center for Biotechnology Information gene database http://www.ncbi.nlm.nih.gov/gene/)	As	Pb	Mn	Cd	Top Ten Interacting? Polymorphism and Increased Susceptibility?	Documented Outcome
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.	-	-	+	-		Intellectual disability, autism
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, Parkinson's, MAPK signaling pathway. Signal transduction in autism.	+	+	+	+	Mn, Cd	Learning disorders
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	Autism
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	Iron metabolism disorders; learning disorders
IL1B	Encodes 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.	+	+	+	+		Learning disorders
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	Learning and cognition disorders
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	Learning and cognition disorders
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.	-	+	+	+		Autism
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.	-	-	-	+		Learning disorders

“+” indicates increased expression; “-” indicates decreased expression; “var” indicates data show both increased and decreased expression

Table VI

Evidence for Combined Effect of Metals in Neurotoxicity (Foundation for an Adverse Outcome Pathway)

1. Support for Biological Plausibility	Strong	Moderate
MIE: Activation of MEK1/2, ERK in astrocytes	As-Cd-Pb: 63 As: 116 Cd: 117,118,138 Mn: 119-121 Pb: 122	
KE: Increased cellular Ca ²⁺	As-Cd-Pb: 60 As: 130,153 Cd: 92,118,134,138 Mn: 120 Pb: 96,97	
KE: Reactive organic species and oxidative stress in brain tissue		As-Mn-Pb: 68 As-Cd-Pb: 63,64 Cd-Pb: 42 As-Pb: 55 As: 116,123,129,130,137,151-153 Cd: 92,117,138 Mn: 98,118,119,134,135 Pb: 97,133
KE: Apoptosis of astrocytes or neurons	As-Cd-Pb: 60-63 As-Pb: 55 As: 116,123,150-152 Cd: 92,118,134,138 Mn: 125,119,120,141 Pb: 96,102,139	
KE2: Disruptions in neurotransmitter release		Cd-Mn-Pb: 33 Cd-Pb: 38,40,47 As-Pb: 54 Cd-Mn: 59 Mn-Pb: 50 As: 129,130,135 Cd: 118,138 Mn: 98,99,133 Pb: 96,97,102,133,139
AO: Learning and cognition disorders		As-Pb: 81,84 reported qualitative interaction, 76-79 reported synergistic Mn-Pb: 14,87 As-Mn: 15
2. Support for Essentiality of KEs	Strong	Moderate
MIE: Activation of MEK1/2, ERK in astrocytes	103, 154, 155	
KE: Increased cellular Ca ²⁺	142, 143, 144, 145, 157	
KE: Reactive organic species and oxidative stress in brain tissue	107, 141	
KE: Apoptosis of astrocytes or neurons	104,128	
KE2: Disruptions in neurotransmitter release	101, 136, 164	
3. Empirical Support for KERs	Strong	Moderate
Key Event Relationships	Empirical support is insufficient to develop regulatory values for synergistic effects of metal mixtures.	

Numbers refer to reference number