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ASSOCIATIONS BETWEEN FEEDING MODE AND HAIR MANGANESE CONCENTRATIONS IN INFANTS

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by

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ABSTRACT

Manganese (Mn) is an essential nutrient, required for growth, development, and health. However, at toxic levels Mn has been shown to lead to cognitive and behavioral deficits in infants and children. Mn toxicity from environmental exposures such as air and water has been associated with decreased intelligence quotient (IQ), externalizing and internalizing behaviors, hyperactivity, and impulsivity. Infant formulas are highly concentrated sources of Mn, containing as much as 50-80 times the amount of Mn as human breast milk. Despite the extreme differences in Mn concentration between infant formula and breast milk, formula has not been well studied as a source of exposure to Mn. The present study investigated Mn status in formula fed and breastfed infants to determine the deposition of Mn from dietary sources in hair.

Infants 3-6 months of age and their mothers were recruited from State College, Pennsylvania, and the surrounding area. Infants were either predominantly breastfed (n=34) or predominantly formula fed (n=19). Hair samples were collected from mothers and infants, and were analyzed for Mn, arsenic (As), cadmium (Cd), and lead (Pb). Mothers provided tap water samples from home, which were analyzed for Mn, As, Cd, and Pb. Infant formula samples were also collected for infants who were formula fed, and were analyzed for Mn.

Infant and maternal hair concentrations were within normal ranges for Mn, As, and Cd. Infants had slightly elevated Pb as measured in hair (mean: 6.22 μg/g), but mothers did not (mean: 0.34 μg/g). Tap water samples were not elevated in Mn, As, Cd, or Pb compared to acceptable limits, though Pb concentration was significantly higher in tap water samples from breastfed infants than from formula fed infants (means: 1.77 vs. 0.82 μg/g, respectively). Infants who were breastfed did not differ in hair Mn concentrations from infants who were formula fed. Salient predictors of maternal hair Mn concentrations included water Mn concentration, employment status, and education. Maternal hair Mn concentration and infant hair As

concentration were significant predictors of infant hair Mn concentration in the fully adjusted regression model. The present findings suggest that ingestion may not be an important exposure mechanism for Mn toxicity in infants compared to environmental exposures. Future studies should investigate co-exposure of Mn and As in central Pennsylvania, and should expand upon present findings indicating limited impact of dietary exposure to Mn during infancy.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 1

Introduction

Manganese (Mn) is an essential nutrient, required for growth, development, and health in humans, animals, and plants. It is necessary for nervous, immune, and reproductive systems functioning, for skeletal development, and for energy metabolism. Activation of several enzymes is dependent on Mn, including those involved in antioxidant function and neurotransmitter synthesis and metabolism (Santamaria 2008). Mn-dependent metalloenzymes include arginase, glutamine synthetase, phosphoenolpyruvate decarboxylase, and manganese superoxide dismutase (IOM 2001).

Diet is one of the most important sources of Mn for the general population, although exposure can occur from fuel (as methylcyclopentadienyl manganese tricarbonyl), fungicides, and contrast images used in magnetic resonance imaging (Aschner 2005). Sources including nuts, legumes, tea, grains, vegetables, and fruits contain the highest amounts of Mn in the normal adult diet in the United States (Pennington 1996). Additionally, infant formulas have high Mn concentrations compared to human breast milk. Cows' milk formula contains 50-100 µg Mn/L compared to human milk at 3-8 µg Mn/L (Lönnerdal 1994). Soy protein is very high in Mn; as such, soy-based infant formula contains a particularly high concentration of Mn, containing up to 200-300 µg Mn/L (Lönnerdal 1994). Mn is also present in drinking water (typically at low levels) and in ambient air (Santamaria 2008).

Absorption of Mn from the diet occurs in the divalent and trivalent state of the metal (Santamaria 2008). The mechanisms of Mn absorption are not well understood. There are two lines of evidence: one supports active transport process by way of the divalent metal transporter 1 (DMT1) (Garcia-Aranda 1983), while others have demonstrated simple passive diffusion (Bell 1989). Mn is taken up from the blood by the liver and transported to extrahepatic tissues by

transferrin (in trivalent form) (Davidsson 1989, Aisen 1969), albumin (Davis 1992), α2 macroglobulin (Rabin 1993), or β-macroglobulin (Critchfield 1992), or can exist in a free dissolved state (Aschner 2005). Several mechanisms for transport into cells have been identified, and may vary based on cell type. Transferrin-bound Mn^{3+} initially binds to the transferrin receptor, which is internalized by the cell in a vesicle. Mn^{3+} reduces to Mn^{2+} (mechanism yet unknown), and crosses into the intracellular space by DMT1. It is notable that transferrin receptors are dense in the globus pallidus, thalamic nuclei, and substantia nigra, which are areas where Mn accumulates in adults in toxic situations (Erikson 2007). Alternatively, Mn can be taken up by DMT1 independent of transferrin. There is also recent evidence of transport via voltage-gated and store-operated Ca^{2+} channels and ionotropic glutamate receptor Ca^{2+} channels (Roth 2006). Distribution of Mn in bodily tissues is fairly homogenous, though bone, liver, pancreas, and kidney tend to have higher levels than other tissues (Keen 1994, Rehnberg 1980). Hair accumulates Mn as well, though the mechanism for this incorporation is not well elucidated. In a toxic situation, Mn tends to accumulate especially in the liver and brain (IOM 2001). Mn is regulated at the point of elimination as well as absorption. The liver is vital for Mn regulation, as the bile system is the main pathway for excretion, with the majority of excretion occurring in feces (Davis 1993, Malecki 1996, Papavasiliou 1966).

Absorption of Mn from dietary sources has been shown to be highly variable. On average, adults only absorb 1-5% of Mn ingested under normal conditions (Finley 1994, Davidsson 1988, ASTDR 2000, Dobson 2004), with women absorbing a greater proportion of the amount ingested (Davidsson 1988). Absorption is lower when a large amount of Mn is ingested together (Britton 1966, Malecki 1996, Finley 1999, Davis 1993). The metal interacts with other micronutrients as well. For example, calcium tends to decrease Mn absorption, and a Mn deficit increases bioavailability of calcium, zinc, iron, copper, and selenium (Davidsson 1991, Planells 2000). Iron status plays a large role; iron deficiency increases absorption of Mn in the gut and

transport of the metal to the brain. There is an inverse relationship between iron stores and Mn absorption as well, likely due to competition for uptake by DMT1 (Davis 1992, Chandra 1976, Shukla 1989, Erikson & Shihabi 2002, Yin 2010). Age also plays a role in the bioavailability of ingested Mn, with increasing age decreasing absorptive capacity (Keen 1986, Dorner 1989). Infants absorb a very high percentage of Mn ingested; neonatal rats were shown to absorb 70-80% of Mn ingested, compared to 1-2% in adult rats (Keen 1986). In addition to different absorptive abilities, gender and age affects Mn requirements, as evidenced by the Institute of Medicine (IOM) guidelines for Mn intake (Appendix D, IOM 2001).

Manganese Neurotoxicity

While it is an essential nutrient, Mn has been shown to act as a toxicant when exposure occurs at high levels. In adulthood, the effects of Mn at toxic levels have been defined as a neurological syndrome with features similar to Parkinson's disease which has been termed "manganism." Manganism is characterized by neuropsychological and neurobehavioral abnormalities that increase in severity with increasing exposure. Subclinical indications may include anorexia, apathy, headaches, joint pain, weakness, insomnia, muscle spasms, and irritability. As exposure increases, toxicity manifests as tremor in the extremities, disturbance of gait, bradykinesia, impaired speech, decreased motor function, mood alterations, decreased hand steadiness, decreased hand-eye coordination, decreased response speed, paresthesia and decreased memory (Santamaria et al. 2007, Checkoway 2010).

Mn toxicity was first demonstrated in miners and ore crushers who were exposed via inhalation of the metal (Rodier 1955, Couper 1837). Since, there have been many reports of occupational exposure to high levels of Mn through mining, welding, and manufacturing of steel, glass, and dry cell batteries (Santamaria et al. 2007, Checkoway 2010). Prior to the 1950s,

scientific reports of exposure were limited to case studies and clinical publications that suggested high concentrations of Mn in dust were responsible for adverse neurologic effects (Santamaria 2007). Research became more focused when occupational exposures were identified as extremely high sources of Mn. Workplace guidelines for Mn were often exceeded for miners, plant workers, and ore-crushers (Tepper 1961). Those working with raw material in a dusty industrial environment tended to have highest exposures, with fewer neurotoxicity reports from battery workers compared to miners and millers (Pal 1999, Tepper 1961). In mining populations, manganism was reported at exposures ranging from as low as 2 mg Mn/m^3 to 20 mg Mn/m^3, in workers exposed for various periods of time (Rodier 1955, Schuler 1957, Witlock 1966, Tanaka 1969, Cook 1974, Saric 1977). Some cases reported onset of symptoms occurring up to 3 years after cessation of exposure (Cook 1974). Studies of exposed workers have reported a high prevalence of headache, anorexia, memory loss, and fatigue in extremely high exposures, along with tremors, muscle rigidity, difficulty with balance, and muscle spasms (Tanaka 1969, Smyth 1973, Penalver 1955). Manganism and neuropsychological effects were well established in occupational environments with extremely high Mn, up to 350 mg Mn/m^3 where ventilation was not present (Smyth 1973).

More recently lower levels of occupational exposure have been investigated, primarily in the welding industry (Santamaria 2007). Cases of neurotoxicity or manganism have occurred in occupational exposures of greater than 5 mg/m^3, with symptoms including somnolence, imbalance, slurred speech, impaired fine movements, bradykinesia, and posture instability (Cook 1974, Rodier 1955, Huang 1989, Santamaria 2008). A case-control analysis of welders exposed to Mn in welding fume demonstrated impaired psychomotor tasks and increased tremor compared to unexposed referents (Ellingsen 2008). A meta-analysis by Lees-Haley and colleagues (2006) analyzed 20 studies of cognitive, psychological, motor, and sensory effects of exposure to Mn. The authors concluded that occupational exposures at levels that are typical (mean air exposure of

3.38 mg/m^3) may have a small effect on cognitive and sensory abilities, but this effect is too small to be detected on an individual basis. A follow-up meta-analysis by Greiffenstein and Lees-Haley looked at neuropsychological variables from 19 studies of occupational exposure (2007). The authors report conflicts in the literature due to poor ability to compare neuropsychological testing and populations between studies. Overall, no relationship was found between Mn in blood, urine, or air and neuropsychological function. It was concluded that confounding variables are better predictors of neuromotor or cognitive dysfunction than Mn exposure in the pooled analysis. Based on these and other studies, the U.S. Environmental Protection Agency (EPA) set a reference concentration at 0.05 μg Mn/m^3 cumulative over one day (Roels 1992, EPA 2002).

Despite conflicting evidence of negative effects of lower levels of Mn, occupational exposures to the metal at levels commonly seen in industrial areas are well accepted as a route to neurotoxicity and manganism. This type of exposure occurs by inhalation, which is particularly critical given evidence suggesting that olfactory uptake may result in Mn-induced neurotoxicity (Henriksson 2000). Mn is taken up into the intranasal space, directly transported to the nasal olfactory bulb by axoplasmic transport, and translocated by secondary olfactory neurons in the brain (Dorman 2002). In the brain, Mn can continue to move along neuronal processes and synaptic networks connected to the olfactory center in the brain (Tjälve 1999). When inhaled, it has also been shown to be transported along the trigeminal nerve into the brain (Lewis 2004).

Exposure to Mn can also occur by ingestion of particles. Dermal exposures are negligible (ATSDR 2000). Of sources of toxic exposure, ingestion has long been considered less important than inhalation due to the limited ability of the human gastrointestinal tract to absorb Mn (Santamaria 2007). However, there has been documentation of adverse symptoms following ingestion of Mn in drinking water either in high levels or by chronic exposures. High concentrations of Mn in drinking water were linked to muscle rigidity, tremors and mental disturbances in six Japanese families (Kawamura 1941). Elderly residents in Greece displayed

adverse neurological effects with chronic exposure as well (Kondakis 1989). A study of people living in a mining district of Mexico, found that Mn concentrations in air were associated with lowered scores on motor tests (Rodríguez-Agudelo 2006). However, a study in Germany found no effects of exposure when comparing adults with chronic exposure to Mn in well water to unexposed controls (Vieregge 1995). Inconsistencies may be due to differences in protocols between studies.

While no studies in adults have found toxic exposure of Mn by ingestion of foods high in Mn, patients experiencing liver dysfunction or renal failure have shown signs of Mn neurotoxicity due to lack of proper regulation, elimination, and clearing of Mn from the blood (Ikeda 2000, Pal 1999 Spahr 1996). Similarly, studies of individuals dependent upon enteral or total parenteral nutrition (TPN) have demonstrated effects of excess Mn exposure. Several publications report Mn accumulation in brain tissue as detected by magnetic resonance imaging (MRI), as increased T1 signaling has been shown to be a sensitive marker for Mn accumulation in regions of the brain (Maeda 1997). One such group found elevated red blood cell Mn concentrations in 71.4% of patients receiving TPN for more than 37 days (15 of 21 patients), including two patients who had elevated red blood cell Mn after just 14 and 18 days of TPN. Case studies of three of these patients in which MRI scans of several brain regions were conducted, and resulted in two patients with T1 hyperintensity in the globus pallidus region (Fitzgerald 1999). Another study retrospectively identified patients diagnosed with Mn neurotoxicity, finding T1 hyperintensity in the basal ganglia along with elevated serum Mn. Cognitive problems including memory loss, difficulty concentrating, cognitive slowing, difficulty choosing words in speech, and spatial disorientation were reported by 13 of 30 diagnosed patients (Klos 2006). A case report of two elderly patients (68 and 70 years) described gait disturbance, psychiatric symptoms, dysarthria, rigidity, tremor, and confusion. Further investigation for both patients revealed MRI T1 signal

hyperintensity in the basal ganglia, especially the globus pallidus, along with elevated serum and urine Mn concentrations (Nagatomo 1999).

Not only have occupational, water ingestion, and TPN exposures been shown to lead to potential neurotoxic outcomes, but ambient environmental exposures have led to similar results. Adults in a risk assessment study in central Mexico who were living within a Mn mining district were shown to have elevated blood Mn and Pb. Exposed adults were at 12 times greater risk of poor cognitive performance on a neuropsychological examination compared to non-exposed referents (Santos-Burgoa 2001).

Manganese Toxicity in Infants and Children

Children may be at particularly high risk for Mn toxicity for several reasons (Menezes-Filho 2009, Winder 2010). First, when ingested, intestinal absorption of the nutrient is higher than in adults (Dorner 1989). Second, a high demand for iron due to high rates of growth may enhance Mn absorption (Mena 1969). Third, low excretion rates occur in infants and young children due to poorly developed biliary excretion (Cotzias 1976).

TPN in infants and children has shown accumulation of Mn, leading to toxicity in some cases. Patients ranging from 1 month to 17 years of age receiving TPN who had elevated serum bilirubin indicative of cholestatic liver disease had elevated plasma Mn levels, correlated with elevated serum bilirubin. This exemplifies the susceptibility of patients on TPN to Mn toxicity due to lack of liver regulation of the nutrient (Hambridge 1989). Another group of children ranging from 6 months to 10 years of age who had been on TPN for either 3 months or over two years were assessed using MRI. High T1 signal was seen in 3 of 4 children on short-term TPN, and 4 of 9 children on long-term TPN. Of all children receiving nutrition support, 69% had elevated whole blood Mn levels, and two reported neurotoxicity evidenced by seizures and

abnormal posturing (Quaghebeur 1996). Fell and colleagues similarly showed high-intensity T1 signaling in the globus pallidus and subthalamic nuclei in a patient with disordered movement with extremely elevated Mn in whole blood (101.1 µg/L), indicating Mn accumulation and likelihood of damage in those brain regions (1996). Evidence from TPN studies has provided ample reason to suggest frequent monitoring of patients for elevated Mn, and the recommendation to minimize Mn supplementation in pediatric and long-term patients (Hardy 2009). Studies of Mn toxicity from TPN cases provide important insight into the impact of poor Mn regulation by the biliary system, and the deposition of the metal in various brain regions when in excess.

Some studies of environmental exposure in infants and children have found important effects on behavioral, motor, and cognitive development (Menezes-Filho, Bouchard et al. 2009). Cognitive and developmental deficits related to Mn exposure have been seen in various populations. Children living near a ferro-manganese alloy plant outside of Salvador, Brazil were found to have elevated Mn concentrations in blood and hair (ages 6-12 years, blood Mn = 8.2 µg/L, hair Mn = 5.83 µg/g), which were significantly higher than non-exposed children of similar socio-economic status (Menezes-Filho, Paes et al. 2009). Hair Mn was negatively related to full-scale and verbal intelligence quotient (IQ) when adjusted for maternal education and nutrition status (Menezes-Filho 2011). In Bangladesh, well water averaging 793 µg Mn/L (compared to the EPA recommended upper limit of 50 µg Mn/L, US EPA 2004) was associated with reduced performance, verbal and full-scale raw IQ scores (Wasserman 2006). An expanded study from this same population revealed reduced perceptual reasoning and working memory associated with elevated blood Mn, even when adjusted for socio-demographic variables, iron status, and arsenic (As) exposure (Wasserman 2011). All three IQ components (verbal, performance, full-scale) were also inversely associated with hair Mn concentration (median = 12.6 µg/g) in 7-11 year old children from central Mexico, with a significant modification by age and sex (Riojas-Rodrigues

2010). Similarly, a cross-sectional study in Korea found a significant linear relationship between Mn and verbal IQ score in children ages 8-11 years. This study also looked at lead (Pb) and IQ, finding that there may be additive interaction and effect modification between Mn and Pb (Kim 2009). More recently, 6-13 year old children in Québec who were exposed to Mn by home tap water (median concentration = 34 µg/L) demonstrated a decrease of 2.4 IQ points for each 10-fold increase in water Mn concentration. Hair Mn concentration was significantly associated with Mn intake from water, and was also significantly associated with lower full-scale and performance IQ scores (Bouchard 2011). Other studies have found similar relationships between IQ and Mn (Wright 2006). Only one study has been done on infants and toddlers, looking at development at 12 and 24 months of age (Claus Henn 2010). A non-linear relationship was found between developmental scores and blood Mn concentrations; the highest and lowest quartile of Mn had significantly lower scores at 12 months of age. This effect was diminished with age, and was no longer significant at 24 months. This vast array of studies puts forth strong evidence for the negative impact of environmental exposure to Mn on cognition and development.

Toxic Mn exposure has been shown to impact behavior in children as well. A preliminary study on hair metals and behavior in childhood found hyperactive children had significantly higher hair Mn concentrations than a group of control children with no behavioral problems diagnosed by their doctors (Barlow 1983). A study in the United Arab Emirates found that school-aged children with attention deficit hyperactivity disorder (ADHD) had significantly higher blood levels of Mn than controls, and that for each 1 ppb increase in blood Mn the odds ratio of ADHD increased by 80.1% (Yousef 2011). Several populations mentioned above have also been involved in studies looking at behavioral outcomes. The population in Québec demonstrated hyperactive effects of Mn exposure through tap water (Bouchard 2006). Hair Mn concentrations (mean of 6.2 µg/g in exposed, 3.3 µg/g in unexposed children) were significantly

associated with teacher ratings of oppositional and hyperactive behaviors in the classroom. In Bangladeshi children, water Mn levels were positively and significantly associated with teacher reported internalizing (anxious/depressed and withdrawn) and externalizing (attention problems and aggressive) behaviors (Khan 2011). Two studies have used tooth enamel as an indicator of prenatal exposure to Mn. One such study used prenatal and postnatal enamel regions of children with Autism Spectrum Disorders, high levels of disruptive behavior, and typically developing children. There were no significant differences in Mn between children with Autism or children with disruptive behavior and typically developing children (Abdullah 2011). In contrast, Mn deposits in tooth enamel from participants in the National Institute of Child Health and Human Development (NICHD) Study of Early Child Care and Youth Development (SECCYD) dating to the 20th week of gestation was associated with behavioral disinhibition, impulsivity, externalizing and attention problems, and disruptive behavior. Mn in tooth enamel at weeks 22-24 after birth was also associated with externalizing behaviors (Ericson 2007). While the impact of Mn exposure in utero is yet to be confirmed, there are clear relationships between environmental exposures to Mn and behavioral problems in childhood.

While environmental exposure and exposure via TPN have been clearly demonstrated as mechanisms for Mn toxicity, dietary exposure has rarely been investigated. Given the higher absorptive capacity of infants and children for Mn coupled with poorly developed biliary excretion in infants up to approximately 6 months of age, ingestion may be an important source of Mn as a toxicant (Winder 2010, Crinella 2003, Cotzias 1976). Infant formula may pose a particular threat, as formula can contain 50.0-300.0 µg/L according to some sources compared to 3-8 µg/L in breast milk (Lönnerdal 1994). Soy formula in particular is high in Mn, containing as much as 50-80 times more of the metal than human breast milk (Cockell 2004, Lönnerdal 1983). Such high amounts of the toxicant are of great concern, given the popularity of using infant

formula as a nutrient source for infants. In 2002, infant formula constituted 56.1% of energy in infants 4-5 months of age, and 43.1% of energy for infants 6-11 months of age (Fox 2006).

An early study investigating the relationship between infant formula and Mn toxicity showed that despite differences in daily Mn intake between breastfed infants and formula fed infants (0.42 µg/kg/day vs. 183.2 µg/kg/day, respectively), mean serum Mn concentrations were similar (Stastny 1984). In contrast, a study assessing body load of Mn from formula found infants fed with formula had significantly higher Mn levels in hair compared to normal ranges at various time points throughout infancy and early childhood, and showed older children with ADHD had almost two times the concentration of Mn in hair than non-ADHD controls (Collip 1983). Breastfed infant hair Mn concentrations were measured at 4 months of age only, and were shown to be significantly lower than concentrations found in formula fed infants. This study, while significant for initial findings, only compared breastfed and formula fed infants at one time point, had a very small sample size for this comparison (10 infants in each feeding group), did not take into consideration mixed feeding modes, and had limited data on potential covariates.

Few animal studies have found cognitive and behavioral insults as a result of Mn from formula feeding. A study of neonatal rats supplemented with 0, 50, 250, or 500 µg Mn/day from birth (mimicking intake from infant formulas) found Mn supplementation led to neurodevelopmental delays, particularly in passive avoidance tests. Supplemented rats had more Mn accumulated in the brain, and also exhibited an inverse relationship between supplementation level and striatal dopamine concentration (Tran 2002). In a study of non-human primates fed cow's formula, soy formula, or soy formula with added Mn, the soy groups demonstrated more internalizing behavior and impulsivity. These groups also had fewer periods of inactivity, relating to shortened attention and hyperactivity. The highest Mn intake group also showed a blunted dopamine response (Golub 2005). While animal studies investigating the effects of Mn in infant formula on behavior and development are important for understanding the potential

impact of this mode of exposure, more human studies must be done to determine the impact of toxic Mn by ingestion during and beyond infancy.

Mechanisms of Manganese Neurotoxicity

Although effects of Mn toxicity have been clearly shown in children and adults, the mechanisms underlying toxicity are not well elucidated. Mn must cross the blood brain barrier in order to accumulate in the brain (striatum, globus pallidus, subastantia nigra, and subthalamic nuclei). There appears to be several mechanisms at work in this process (Erikson 2007). Facilitated diffusion, unidirectional active transport, DMT-1 mediated transport, ZIP8 transporter, store-operated calcium channels, and transferrin-dependent transport all have been shown to be involved (Schmitt 2011, Rabin 1993, Aschner 1994, Erikson 2004, Erikson 2007). It is still unknown which mechanism predominates in humans.

Oxidation has been widely accepted as a contributing pathway of effect of toxic Mn exposure. However the specific components of the mechanism are not well established. It is likely that Mn acts in a variety of ways to impact the brain to cause cognitive, behavioral, and psychomotor effects. One potential mechanism of action is the oxidation of dopamine by Mn, potentially as a result of reactive oxygen species (ROS). ROS and free radicals could be formed by nonenzymatic autoxidation of catecholamines or production of 6-hydroxydopamine (Erikson 2007). Oxidation of dopamine has been supported by studies showing Mn accumulation in dopamine-rich areas of the brain in rodents and primates after chronic exposure (Sloot 1996). Additionally, Mn can inhibit mitochondrial enzymes, interfering with respiration which can lead to excessive production of ROS. There is evidence of inhibition of both complex I and ATPase complex of the electron transport chain in the presence of mitochondrial Mn (Brouillet 1993, Gavin 1999). Although the divalent form is much more prevalent within cells and is found bound to ATP, the trivalent form is more effective at complex I inhibition (Ali 1995, Gunter 2002).

Notably, a small amount of Mn^{3+} will be formed spontaneously from any valence form of Mn, and trace amounts of Mn^{3+} can cause ROS formation (HaMai 2001).

Another potential mechanism involves ROS inhibition of glutamate transporters, thus limiting glutamate removal in neurons (Trotti 1998). This causes an increase in extracellular glutamate, which may be excitotoxic to neurons. This mechanism has been supported by studies showing that Mn-exposed astrocytes exhibit reduced glutamate uptake and a reduction in GLAST (gene that encodes for glial high affinity glutamate transporters) gene expression, though this has yet to be confirmed in vivo (Erikson & Aschner 2002, Erikson & Suber 2002).

More recently these mechanisms have been studied in the context of the neonatal brain, showing that neonates may respond differently to Mn toxicity. It seems that the striatum is the main region impacted in neonates, while the hippocampus, midbrain, and olfactory bulb show effects of toxicity in adults (Erikson 2007). Neonatal rats exposed to Mn had significantly reduced antioxidant function (measured by glutathione) and gene expression of metallothionein and glutamine synthase (markers of oxidative stress) in the striatum (Erikson 2006). Another study showed that neonatal rat striatum was more susceptible to Mn toxicity than older rats (Erikson 2004). It has also been shown that dopamine and GABA changes were most drastic in the striatum in rats exposed to Mn from birth (Tran 2002).

While several mechanisms have been proposed to explain the effects of Mn toxicity and the differential effects of age on signs and symptoms of exposure, more work needs to be done to fully understand all mechanisms of action, particularly in the neonate and infant.

Rationale

Mn is necessary for normal growth and functioning in all phases of the human life cycle; however only limited amounts are required for bodily processes (Santamaria 2008). Mn is essential for nervous system function, antioxidant function, skeletal growth, energy metabolism, and enzyme activation (Santamaria 2008). The amount of Mn required for these functions can easily be met by normal dietary habits. The Institute of Medicine recommends 0.003 mg Mn/day for infants less than 6 months of age, 0.6 mg/day for infants 7-12 months of age, and amounts ranging from 1.2-2.2 mg/day for children and adolescents from 1-18 years of age depending on age and gender (IOM 2001) (Appendix D). The adequate intake (AI) for infants is based on the amount of Mn provided through breast milk from exclusive breastfeeding for the first 6 months of life. The recommendation increases at 7-12 months of age, in accordance with the introduction of complementary foods.

Infants can easily consume adequate and safe levels of Mn from exclusive breastfeeding. In contrast, infants who are fed infant formula often far exceed the AI for Mn. Formulas are a common feeding choice for infants in the United States, suggesting that a high percentage of infants are consuming amounts of Mn that far surpass the AI, putting them at potential risk for negative effects of Mn neurotoxicity. Given that negative effects of Mn exposure on cognition and psychomotor abilities have been seen in adults as well as cognitive and behavioral development in infants and children, infant formula exposure to Mn must be investigated as a potential cause of Mn toxicity.

Specific Aims

In order to better understand the relationship between Mn exposure through infant formula and behavioral and cognitive outcomes, differences in body Mn accumulation based on primary feeding mode must first be better understood. The present study focused on body Mn levels in infants as measured by hair Mn concentration. The primary objective of the study was to understand this deposition from dietary exposure. This project was designed to pursue the following specific aims:

Aim 1: To determine the extent to which breastfed and formula fed infants 3-6 months of age differ on hair manganese levels.

Hypothesis 1: Infants who are primarily formula fed have significantly higher hair manganese concentrations than infants who are primarily breastfed.

Aim 2: To determine salient predictors of hair manganese concentration in infants 3-6 months of age.

Hypothesis 1: Higher maternal hair manganese concentration is associated with higher infant hair manganese concentration.

Hypothesis 2: Higher water manganese concentration is associated with higher infant hair manganese concentration.

Hypothesis 3: Infant hair manganese concentration increases with infant age.

Hypothesis 4: Higher concentration of manganese in infant formula is associated with higher infant hair manganese concentration in infants who are formula fed.

Chapter 2

Methods

Study Setting

Data were collected on mothers and infants from State College, Pennsylvania. This area is characterized by mostly middle class families with a fairly high education level, as a majority of families are involved in some regard with The Pennsylvania State University.

Participants were recruited From April 2010 through February 2012. Mothers and infants were informed of the study via flyers and advertisements in campus buildings, as well as town shops, cafes, restaurants, churches, community buildings, local WIC offices, and family practice clinics. Daycare providers were given information to send home with mothers of infants. Flyers and a written description of the study were sent home with new parents in discharge packets from the labor and delivery unit, or were provided to pregnant women on prenatal hospital tours at the Mount Nittany Medical Center. Information was posted online at various websites including www.craigslist.org and www.statecollege.com. Study information was made available at the Grange Fair, a week-long highly popular local fair in central Pennsylvania. Recruitment information was provided to individuals involved with Penn State University through the faculty/staff newswire once per semester from spring 2010 through spring 2011, and on weekly lists of events sent to the College of Health and Human Development. Word of mouth and lateral recruitment was utilized by giving enrolled participants additional flyers to pass along to other eligible mothers if they were willing to do so.

Once potential participants showed interest in the study, a set of screening questions was asked over the phone to determine eligibility. Mothers were eligible if they were 18 years or older, spoke English fluently, considered themselves very confident in their infant's feeding and

intake composition, and were able to attend the appointment in the laboratory on Penn State's University Park campus. Infants were eligible if between 3-6 months of age, from a singleton pregnancy, fed primarily (70%) breast milk or infant formula, and without major health problems. Both mother and infant had to be eligible for the study for the pair to participate.

If the mother and infant were eligible to participate, an appointment was scheduled for them to come to the laboratory. A package was mailed to participants including directions to the appointment location, confirmation of appointment date and time, parking information, and sample collection information. Two sterile plastic collection cups were included in the package for tap water and infant formula (if applicable) samples, which the mothers were directed to bring to the appointment. Appointments were held in Noll Laboratory and the General Clinical Research Center on the University Park campus of Penn State University. Parental consent was obtained at the beginning of the study visit. This study protocol was approved by the Institutional Review Board of The Pennsylvania State University.

Socio-Demographic and Feeding Assessment

A questionnaire was completed during the appointment to collect socio-demographic information about the participants. The questions were asked to the mother in an interview format to allow for further explanations if necessary. Infant birth weight, birth length, and gestational age were recorded. Maternal pre-pregnancy BMI was determined by self-reported pre-pregnancy weight and height, and coded for analysis as <24.9, 25-29.9, or >30 kg/m^2. Pregnancy smoking habits were recorded based on whether the woman smoked (yes, no) and the quantity of cigarettes smoked daily (<1, 1-9, 10-19, >20). Current smoking habits were recorded as current smoking status (yes, no) and quantity of cigarettes smoked daily (<1, 1-9, 10-19, >20). Questions asked about maternal education (high school, some college, college, post-graduate),

marital status (married, living with partner, single, divorced, widowed) and current employment status (paid maternity leave, unpaid maternity leave with benefits, unpaid maternity leave with no benefits, employed full time, employed part time, unemployed with no benefits, unemployed with benefits, student, other). Income was categorized into $20,000 brackets to improve confidence in the correct response and to maximize participant comfort in answering the question (<$19,999; $20,000-39,000; $40,000-59,000; $60,000-79,000; $80,000-99,000; >$100,000). Race (American Indian or Alaskan Native, Asian, Black or African American, Native Hawaiian or Other Pacific Islander, White) and ethnicity (Hispanic, non-Hispanic) were asked as open-ended questions to allow participants to report as they felt most appropriate. Additionally, mothers were asked about any hair treatments in the last six months.

An infant feeding questionnaire was also completed during the participant appointment in an interview style. Mothers answered questions on infant health status including doctor diagnosis of gastroesophogeal reflux, dehydration, blood in stool, allergies, or poor weight gain. Infant use of supplements, vitamins, herbs, or medications was reported. Mothers reported monthly feeding mode as an estimated percent of total diet comprised of breast milk or formula for each month since birth, and for each week since three months of age. To gather more information on dietary exposure to manganese, brands and types of formula used for infant feeding were reported. Mothers reported the age at which complementary foods or other beverages were introduced if applicable, and what foods or beverages had been provided to the infant.

Anthropometric Measurements

Infant anthropometric data was collected at participant appointments. All measurements were taken without clothing or diaper. Infant weight was measured to the nearest 1 g using a

digital scale (Seca Corp., Hanover, MD). Weight was measured in duplicate, unless weights differed by more than 10 g in which case a third weight was taken. Weight measurements were averaged for a final weight. Length measurements were taken to the nearest 0.1 cm using an infantometer (Seca Corp., Hanover, MD). Length was measured in duplicate, unless lengths differed by more than 0.5 cm, in which case a third measurement was taken. Infants were measured in the recumbent position with the head positioned in the Frankfurt plane. Length measurements were averaged for a final length.

Sample Collection

Mothers were provided with sterile plastic sample collection cups and were instructed to bring to the appointment 4 oz. of water from the home tap and 4 oz. of infant formula as it is typically made for the infant. Mothers were asked to collect the samples as close to appointment time as possible. All mothers brought a tap water sample to the appointment. Water samples were acidified (pH 2) with concentrated nitric acid (70%) within 4 hours of the appointment and stored at 0°C until analysis. All 19 mothers of formula fed infants brought infant formula samples to the appointment. Of these, 2 samples were presented as formula powder not mixed as the mother typically does, making them unanalyzable. Additionally, one sample was subject to laboratory error in the analysis process, for a total of 16 analyzable infant formula samples. Infant formula samples were collected from mothers at the appointment and immediately stored at 0°C until analysis.

Maternal hair samples were collected from the occipital region of the scalp using sterile stainless steel scissors. Approximately 50 strands of hair were cut as close to the scalp as possible. Infant samples were collected using sterile blunt tip scissors. Hair was cut from various

regions of the scalp depending on where there was enough hair to sample, typically near the nape of the neck. Hair samples were collected in white envelopes, sealed with tape, and stored at room temperature until analysis.

Sample Analysis

For maternal hair samples, the 2 cm closest to the scalp were digested and used for analysis. For infants, the 1 cm closest to the scalp was digested and used for analysis. Hair samples were cut, weighed and placed in sterile plastic vials. For determination of metal levels, samples were washed with 10 mL 1% Triton-X detergent and sonicated for 15 minutes. Samples were rinsed three times with double distilled water and dried in a 60°C oven for 24 hours. 1 mL of concentrated nitric acid (70%) was added to each vile. The vials were placed in an oven at 80°C for 11 hours until hair was fully digested. Each sample was diluted with 20 mL of double distilled water. Digested samples were kept at 0°C.

Hair and water samples were analyzed at the Materials Characterization Laboratory at the Pennsylvania State University (State College, PA). Samples were analyzed by ICP-MS with Collision Cell Technology (Thermo Fisher Scientific X) in 3-5 mL of the solution prepared above for hair or 3-5 mL of water to determine levels of Mn, cadmium (Cd), arsenic (As), and Pb (Limit of Detection: 0.04, 0.01, 0.03, and 0.04 ppb, respectively). NIST1643e trace element in water solution served as the instrument standard, and was used prior to each use (NIST, Gaithersburg, MD). The instrument was externally calibrated based on bias from the standard, which varied from 1.7-4.5% for Mn, 7.8-10.0% for As, 6.4-7.9% for Cd, and 2.9-9.8% for Pb.

Infant formula samples were stored at 0°C until analysis. At the time of analysis, samples were homogenized by shaking, and 2 mL of the formula was pipetted into an acid washed glass vial. For the digestion of proteins and lipid components of the formula, 3 mL

concentrated nitric acid (70%) was added. Vials were loosely covered with plastic wrap and left to digest for 48 hours. Vials were placed on a hot plate, and samples were boiled and reduced in volume to less than 1 mL. Samples were then transferred to a volumetric flask and diluted up to 1 mL with double distilled water. Samples were transferred to plastic vials for analysis. Mn concentration was measured by atomic absorption spectrometry (Perkin Elmer, Waltham, MA) at 279.48 nm wavelength (Feldman 1967) with a 0.5 reading time. A standard curve was made using a 1,000 ppb Mn standard (Fisher Scientific, Pittsburgh, PA) diluted to 0.05, 0.1, 0.2, 0.4, and 0.8 ppb. The standard curve was read into the instrument for calibration prior to sample reading. Each sample was read 3 times and averaged for a mean concentration (coefficient of variation = 1.1%).

Statistical Analysis

Demographic and Feeding Variables

Mothers reported infant feeding mode as the percentage of the diet comprised of breast milk or the percentage comprised of infant formula for each month since birth. Predominant feeding mode was defined as 70% of the diet coming from one source. Infants who consumed at least 70% breast milk were considered predominantly breastfed while those who consumed at least 70% formula since birth were considered predominantly formula fed. Feeding mode was coded as breastfed or formula fed to analyze differences between mother-infant pairs based on type of infant diet.

Demographic variables that had no reported incidents of some possible participant responses were collapsed for analysis. Marital status was recoded as married/living with partner or single, as no other responses were applicable to any participants. Maternal education was recoded as some college or below, college, or post-graduate education. Maternal occupation was

collapsed into employed (which included full-time students receiving a stipend), on maternity leave, or unemployed.

Identification of Outliers

Data was first plotted using a box plot and scatter plot for hair metal concentrations for infants. Infants were plotted as an entire group, and then broken down by feeding mode. In plotting hair metal concentrations for infants, two participants were identified as having very high Mn levels (more than 4 times the standard deviation of the distribution above the mean). Infant hair Mn concentration descriptive statistics were run with and without these two individuals. The mean hair Mn concentration did not change substantially when calculated with or without the potential outliers (2.13 ± 2.68 ppm vs. 1.68 ± 1.39 ppm, respectively). Since there was not a substantial change when removed, and since the values of hair metal concentrations of potential outliers were still well within biological plausibility, they were included in the analysis.

A box plot and scatterplot were constructed for mother hair metal concentrations. In both plots, one participant was a visible outlier in all hair metal concentrations (Mn= 61.96 ppm, As= 0.17 ppm, Cd= 0.0297 ppm, Pb= 0.129 ppm). Means for maternal hair Mn concentration changed substantially when she was removed from the dataset (1.412 ± 8.404 ppm with outlier included compared to 0.248 ± 0.552 with outlier excluded). Since she was a very influential point for hair Mn concentrations in particular, the participant was excluded from the dataset.

Descriptive Statistics

Descriptive statistics were used to explore these data to better understand the population of interest, and to determine differences between breastfed infants and formula fed infants. The mean, standard deviation, and range were determined for child characteristics including age, anthropometrics, and birth information, and for maternal characteristics including age and pre-

pregnancy anthropometrics. Categorical variables were broken down by percentages of participants within each group for infant sex, feeding mode, complementary feeding information, maternal pre-pregnancy BMI, smoking habits, education, marital status, employment, hair treatment, household income, and parent and child race and ethnicity. Normality of the dataset was assessed using a normal probability plot. Hair metal concentrations for infants and mothers were log-transformed to improve normality and fit of the data for linear regression analysis.

Differences between infants who were formula fed and infants who were breastfed were tested using a one-sided nonparametric Wilcoxon Rank Sum test to determine statistical difference for continuous variables and chi-squared testing for categorical variables. Fisher's exact test was used to test categorical variables when there were less than 5 participants in a group. Metal concentrations within and between infant hair, mother hair, water, and formula samples were assessed using Spearman correlation coefficients. A p-value of < 0.05 was considered statistically significant for all statistical tests.

Predictors of Hair Mn Concentration

Bivariate linear regression was used to relate the dependent variable (infant or maternal hair Mn) to potential predictors ($p < 0.15$). Potential predictors were determined based on biological plausibility and a review of the literature, and included for infant hair Mn concentration: age, gestational age, sex, other hair metal concentrations, maternal hair metal concentrations, water metal concentrations, introduction of complementary feeding, primary feeding mode, maternal age, maternal smoking habits, demographic variables including race and ethnicity, income, maternal education, and maternal employment, and anthropometric variables including birth length and weight, current length, weight, and related z-scores, and maternal BMI. Potential predictors of maternal hair Mn concentration included age, other hair metal

concentrations, water metal concentrations, smoking habits, BMI, and demographic variables including race, ethnicity, income, marital status, education, employment.

Multivariate Modeling

Salient predictors for maternal hair Mn concentration that were determined based on bivariate modeling ($p < 0.15$) then were analyzed in a multiple linear model to identify a group of predictive variables. Water Mn concentration, maternal employment, and maternal education were included in the full multivariate model.

Once salient predictors were determined for infant hair Mn concentration ($p < 0.15$ in bivariate models), they were analyzed together in a multiple linear model to identify a group of variables that can be used to predict children's hair Mn levels. Covariates of the full model included infant hair As, Cd, and Pb concentrations, maternal hair Mn and As concentrations, current smoking status and pregnancy smoking status of mother, infant complementary feeding status, child ethnicity, maternal education, and weight-for-length z-score. SAS, version 9.3 (SAS Institute, Cary, NC) was used for all statistical analyses.

Chapter 3

Results

Participant Characteristics

Figure 1 describes the number of people screened for the study, those eligible to participate, and those with complete data. 53 mother/infant pairs were enrolled in the study, with 19 of the infants being predominantly formula fed. Of all included participants, one infant did not have a length measurement completed due to lack of personnel necessary for the protocol, and two infants had hair samples below the limit of detection (LOD) for all metals. One mother felt she was not able to accurately report her pre-pregnancy weight due to uncertainty; this was the mother of one of the infants with hair metal concentrations <LOD. In total, there were 50 mother/infant pairs with complete data.

Infants were approximately 4.6 months of age on average at the time of study appointment (**Table 1**). They tended to have been of normal gestational age at birth, between 38-42 weeks (mean ± SD: 39.5 ± 1.4 weeks). All but 4 mothers (86.8%) reported exclusive feeding by either breast milk or infant formula. More infants were breastfed (64.2%), and about half of all infants had been introduced to complementary foods by study enrollment (45.3%). Almost all infants were non-Hispanic and Caucasian (96.2% and 96.2%, respectively).

Mothers were approximately 30 years old on average (30.8 ± 4.3 years), and tended to have a normal BMI (54.7%). Most mothers had a college or post-graduate education (79.2%), were married (90.6%), and were employed full or part-time (62.3%). Almost all mothers were non-Hispanic and Caucasian (96.2% and 98.1%, respectively). Mothers also reported almost all fathers to be non-Hispanic and Caucasian (98.1% and 96.2%, respectively). Income levels were normally distributed, with 75.5% reporting income of $40,000 or more.

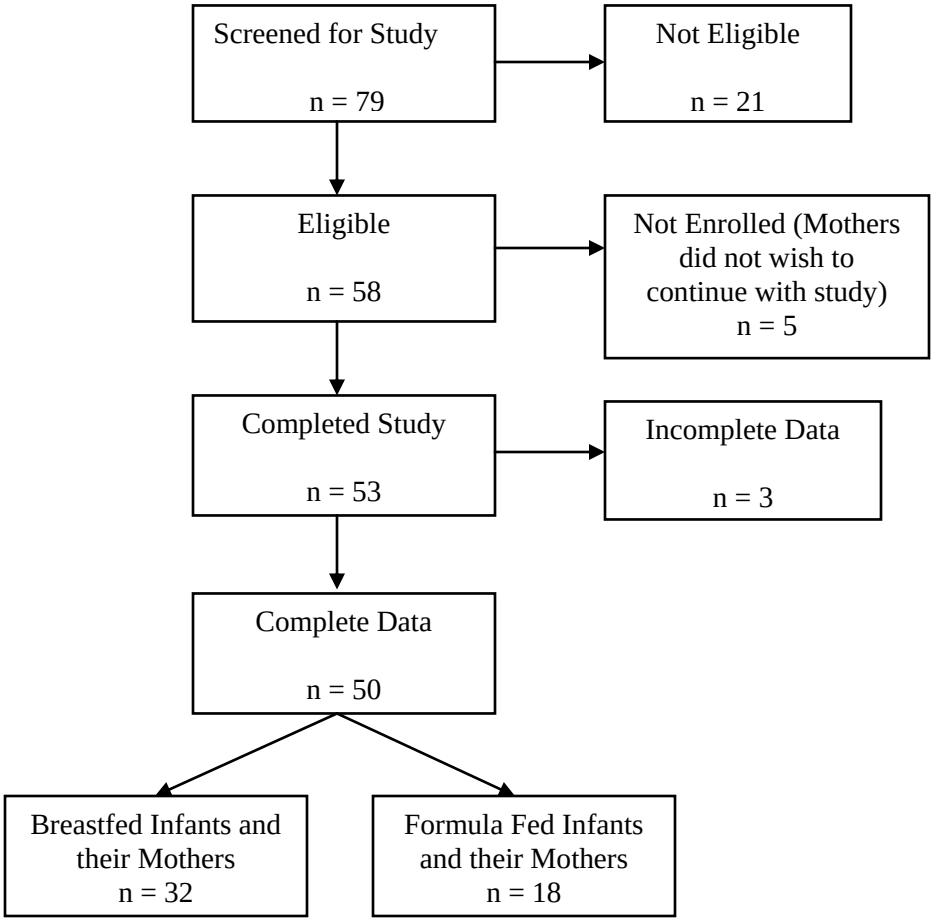

Figure 1. Overview of Study Eligibility, Participation, and Data Completion for Mother/Infant pairs

Table 1. Demographic Characteristics of Study Population

	n	Mean ± SD or %	Range
	Child Characteristics		
Age (months)	53	4.6 ± 1.2	2.7 - 7.0
Sex			
Male	22	41.5%	--
Female	31	58.5%	--
Gestational age (weeks)	53	39.5 ± 1.4	35 - 41.6
Birth weight (g)	53	3423.8 ± 583.4	2211.3 - 5372.2
Birth length (cm)	53	52.4 ± 3.2	45.7 - 59.1
Current weight (g)	53	6745.0 ± 1027.3	4823.0 - 9382.5
Current length (cm)	52	64.1 ± 3.5	56.3 - 71.2
Feeding mode			
Breastfed	34	64.15%	--
Formula fed	19	35.85%	--
Complementary feeding introduced	24	45.30%	--
Age of complementary feeding (weeks)	23	17.5 ± 6.3	2 - 24
Use of medication or supplements	24	45.28%	--
Ethnicity			
Hispanic	2	3.80%	--
Non-Hispanic	51	96.20%	--
Race			
Caucasian	51	96.20%	--
Non-Hispanic black	1	1.90%	--
Asian	1	1.90%	--

(Table 1 continued)

	n	Mean ± SD or %	Range
Mother Characteristics			
Age (years)	53	30.8 ± 4.3	20.5 - 42.0
Height (cm)[1]	53	162.3 ± 21.7	152.4 - 185.4
Pre-pregnancy weight (kg)[1]	52	73.6 ± 22.8	47.6 - 153.8
Pre-pregnancy BMI (kg/m^2)[2]			
Underweight (BMI <18.5)	1	1.89%	--
Normal weight (BMI 18.6-24.9)	29	54.72%	--
Overweight (BMI 25.0-29.9)	8	15.09%	--
Obese (BMI >30.0)	15	28.30%	--
Smoked during pregnancy	2	3.8%	--
Currently smoking	3	5.7%	--
Education			
High school	3	5.7%	--
Some college	8	15.09%	--
College	24	45.28%	--
Post-graduate	18	33.96%	--
Marital Status			
Single	4	7.55%	--
Not married, living with partner	1	1.89%	--
Married	48	90.57%	--
Ethnicity			
Hispanic	2	3.80%	--
Non-Hispanic	51	96.20%	--
Race			
Caucasian	52	1.90%	--
Non-Hispanic black	1	98.10%	--
Employment Status			
Employed full-time	21	39.62%	--
Employed part-time	12	22.64%	--
Student	5	9.43%	--
Maternity leave with no benefits	2	3.77%	--
Unemployed with no benefits	12	22.64%	--
Unemployed with benefits	1	1.89%	--
Hair treatment in previous 6 months[3]	15	28.30%	--

(Table 1 continued)

	n	Mean ± SD or %	Range
	Father Characteristics		
Ethnicity			
Hispanic	1	1.90%	--
Non-Hispanic	52	98.1%	--
Race			
Caucasian	51	96.2%	--
Native American	1	1.90%	--
Asian	1	1.90%	--
	Family/Household Characteristics		
Income			
>$19,999	3	5.66%	--
$20,000-39,999	10	18.87%	--
$40,000-59,999	17	32.08%	--
$60,000-79,999	10	18.87%	--
$80,000-99,999	10	18.87%	--
>$100,000	3	5.66%	--

[1]Height and pre-pregnancy weight are self-reported; [2]Pre-pregnancy BMI based on self-reported height and pre-pregnancy weight; [3]Hair treatments reported included color (n = 14) and permanent wave (n = 1).

Metal Concentrations

Hair Samples

This State College sample of infants and mothers did not exhibit high levels of metal exposures (**Figures 2-9, Table 2**). For all metals, infants had higher mean hair concentrations than mothers. Infants had a mean hair Mn concentration of 2.13 μg/g (SD = 3.29), considered to be within a normal range (Miekeley 1998). Seven infants and two mothers had hair Mn concentrations above 3 μg/g, a proposed cutoff for elevated hair Mn levels (Miekeley 1998). Infant and mother As concentrations were quite low (0.20 ± 0.41 μg/g and 0.03 ± 0.03 μg/g, respectively), with no participants showing a concentration above 7 μg/g, a proposed upper limit for normal hair As concentrations (Pangborn 1994). A proposed upper limit for normal hair Cd concentration is 1 μg/g; two infants and no mothers exceeded this limit (Miekeley 1998). Infants demonstrated slightly elevated hair Pb concentrations, with a mean of 6.22 μg/g (SD = 9.88 μg/g). Using the proposed cutoff of 3 μg/g for Americans, 28 infants (54.9%) had elevated hair Pb concentrations (Park 2006). Of infants with elevated hair Pb, 19 were breastfed (67.9%) and 9 were formula fed (32.1%). All mothers had a Pb concentration within the normal reference range (0.34 ± 0.32 μg/g).

Hair metal levels were typically within the limit of detection (LOD) for Mn and Pb for both mothers and infants, except the two infants who had hair samples below the LOD for all metals due to a very small amount of hair available. For infants, a total of seven participants had As levels below the LOD whereas all mothers had detectable As concentrations. More samples fell below the LOD for Cd than any other metal. A total of 10 infants and eight mothers had undetectable levels of Cd present in the hair samples.

Metal concentrations in infant hair samples were correlated with one another (**Table 3**). Infant hair Mn was strongly correlated with As and Cd, (Spearman's rho = 0.58, 0.46, respectively, $p < 0.01$ for all). Infant hair Mn was also correlated with Pb, but less strongly (Spearman's rho = 0.27, $p < 0.10$). As in infant samples was strongly correlated with Cd (Spearman's rho = 0.45, $p < 0.05$), but not correlated with Pb (Spearman's rho = 0.03). Cd was strongly correlated with Pb in infant hair (Spearman's rho = 0.54, $p < 0.01$).

Mother Mn concentrations were not correlated with any other metal measured in maternal samples. Maternal Pb concentrations were significantly associated with As (Spearman's rho = 0.28, $p < 0.05$) and Cd (Spearman's rho = 0.39, $p < 0.01$). Infant and mother hair metal concentrations were correlated with one another for all metals except As. Water Pb trended towards a correlation with water Cd (Spearman's rho = 0.35, $p < 0.10$). Infant formula samples were not significantly correlated with infant hair Mn concentration.

Water Samples

Water samples were provided by all mother/infant pairs. Mn and Pb were present in detectable concentrations for all samples (**Table 2**). Three samples and 26 samples were below the LOD for As and Cd, respectively.

Water Mn concentrations ranged from 0.04-10.0 µg/L, with a median of 0.21 µg/L and a mean of 0.74 µg/L (SD = 1.66 µg/L). This can be considered very low and is well below the guidelines for maximum amount of Mn in drinking water set at 50 µg/L according to the Environmental Protection Agency (EPA 2004). Mean Pb concentration was 1.43 µg/L (SD = 2.18 µg/L). This is below the WHO guideline for drinking water set at 10.0 µg/L (WHO 2008), though one sample was in excess of this suggested limit with a concentration of 15.0 µg/L. As concentrations were low as well (0.09 ± 0.05 µg/L), with all samples falling below the WHO recommended upper limit of 10 µg/L in drinking water (WHO 2008). Cd levels were low for the

collected samples, evidenced by the low mean concentration (0.07 ± 0.13 µg/L) and additionally supported by the large number of samples falling below the LOD (n = 26). All samples were within normal limits according to the WHO recommendation to keep drinking water Cd less than 3 µg/L (WHO 2008).

Formula Samples

It was estimated prior to formula analysis that infant formula contains approximately 15 µg/ 5 fluid ounces, or 100 µg/L, based on reported Mn levels on various infant formula nutrition labels (**Table 4**). All infant formula samples were above the estimated infant formula Mn concentration based on nutrition labeling for the reported formulas (**Table 2**), ranging from 115-243 µg/L (182 ± 40 µg/L). No mothers reported using a soy-based infant formula.

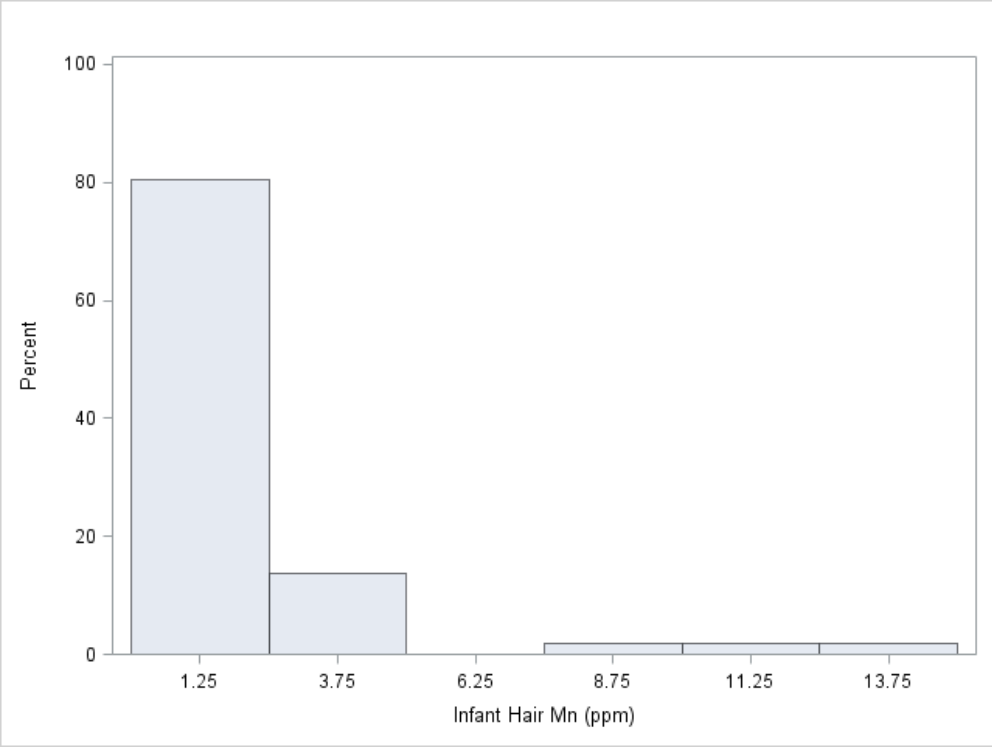

Figure 2. Histogram of infant hair Mn concentration (ppm)

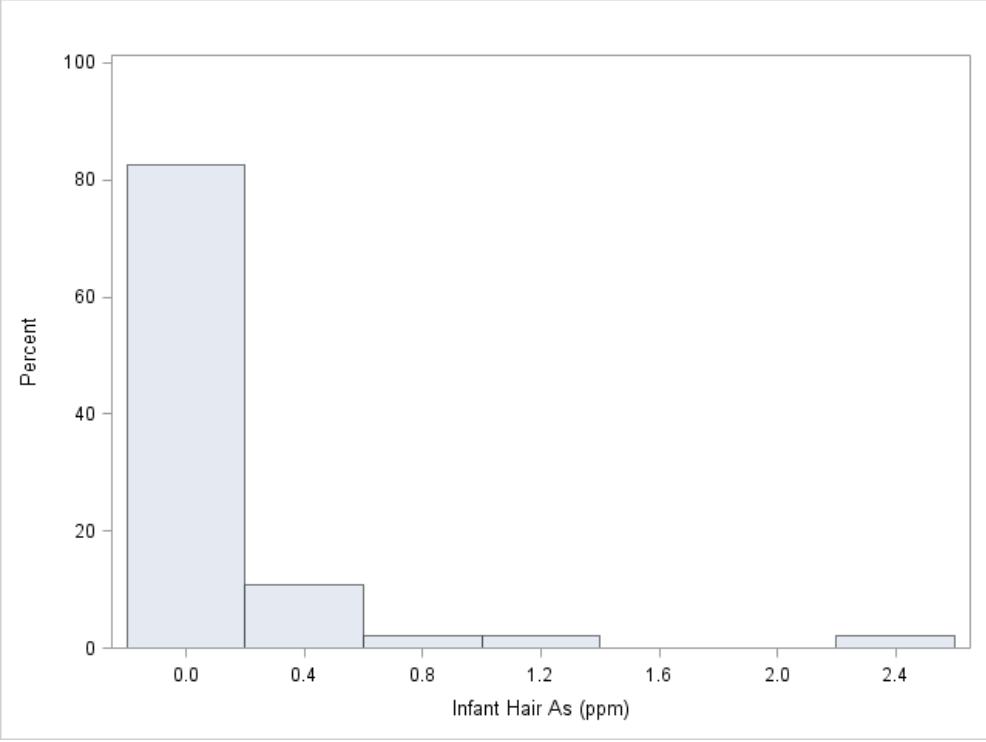

Figure 3. Histogram of infant hair As concentration (ppm)

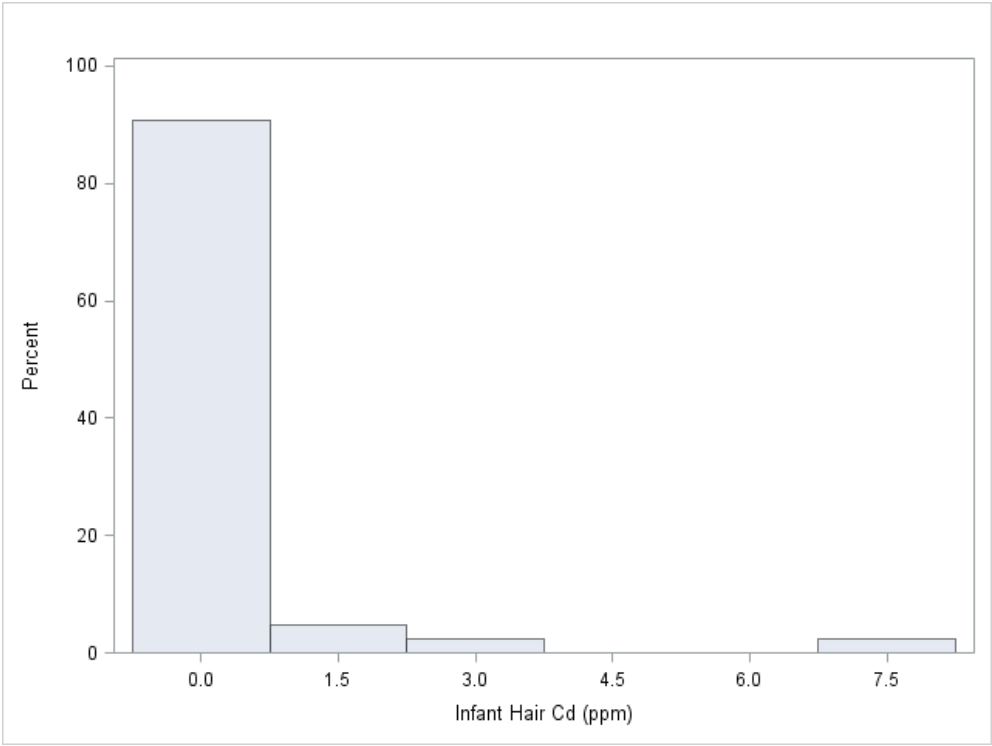

Figure 4. Histogram of infant hair Cd concentration (ppm)

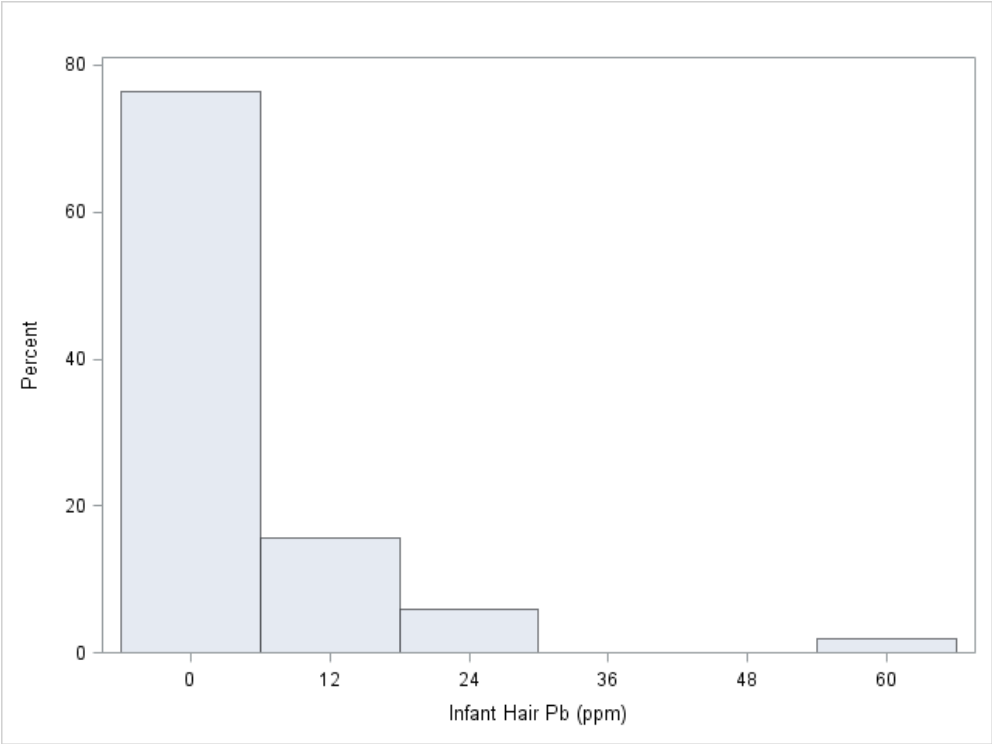

Figure 5. Histogram of infant hair Pb concentration (ppm)

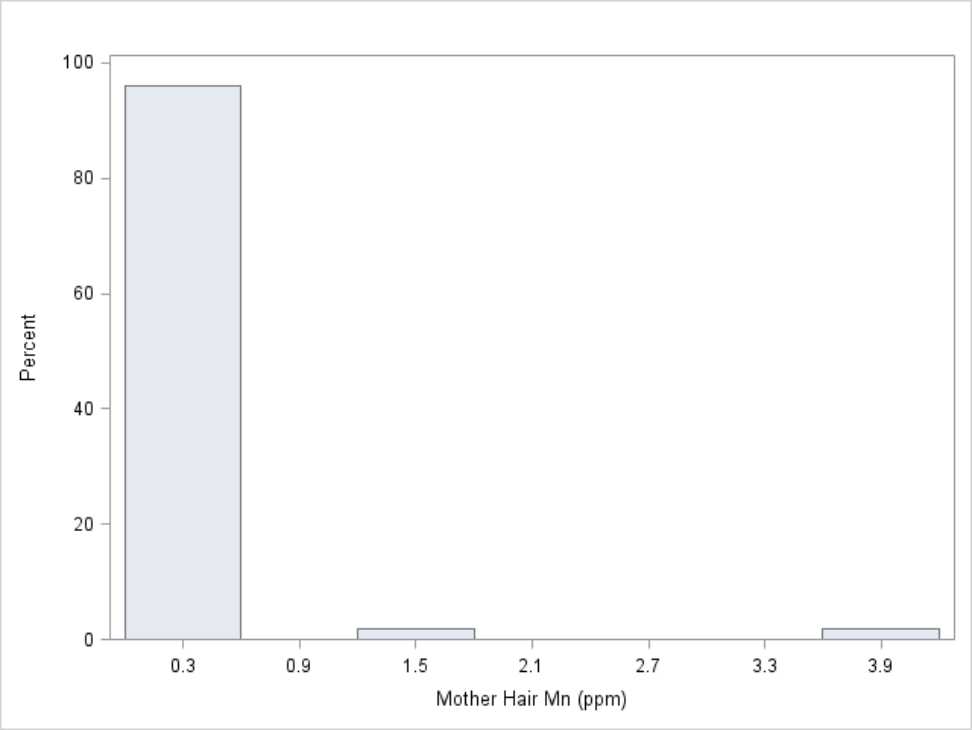

Figure 6. Histogram of mother hair Mn concentration (ppm)

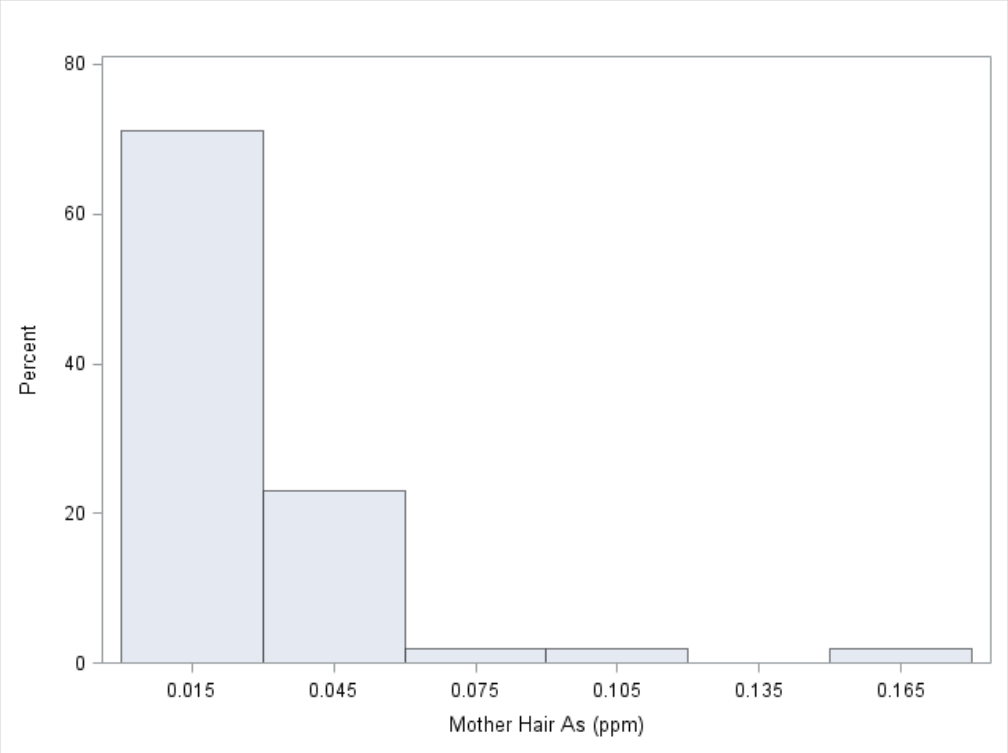

Figure 7. Histogram of mother hair As concentration (ppm)

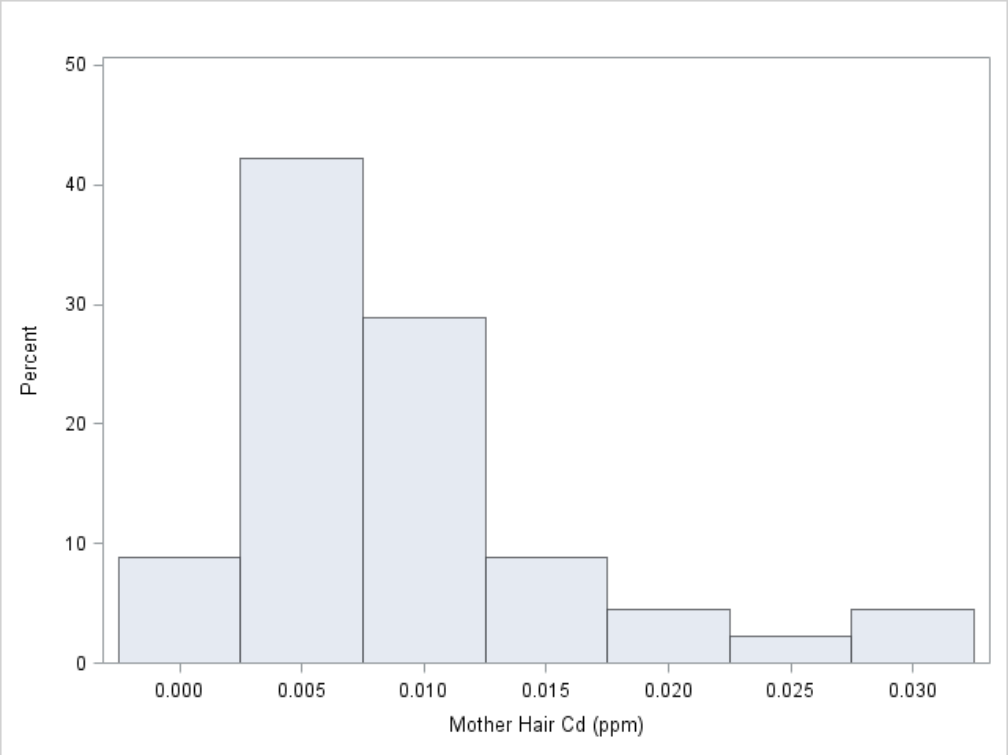

Figure 8. Histogram of mother hair Cd concentration (ppm)

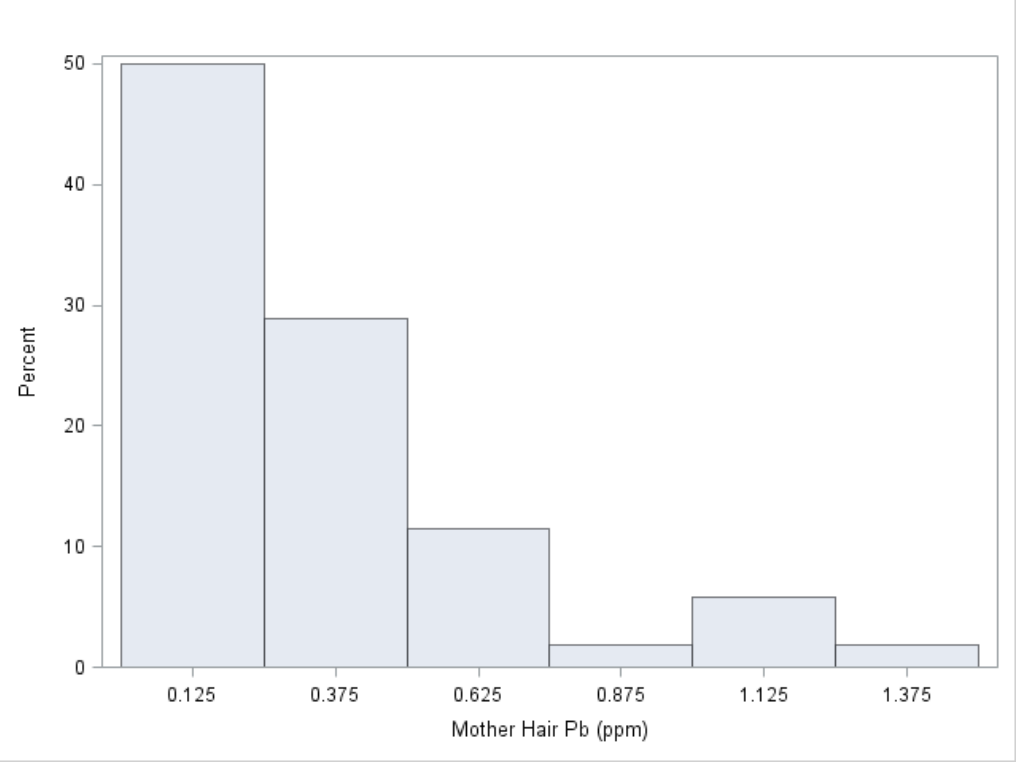

Figure 9. Histogram of mother hair Pb concentration (ppm)

Table 2. Metal concentrations in infant, mother, water, and infant formula samples

	n	Mean ± SD	Median	Range	Acceptable Level
Infant					
Mn (µg/g)	51	2.13 ± 3.29	1.44	0.25 - 14.40	<3.0
As (µg/g)	46	0.20 ± 0.41	0.09	0.007 - 2.40	<7.0
Cd (µg/g)	43	0.48 ± 1.23	0.18	0.02 - 7.67	<1.0
Pb (µg/g)	51	6.22 ± 9.88	3.1	0.08 - 61.60	<3.0
Mother					
Mn (µg/g)	53	1.41 ± 8.49	0.14	0.04 - 61.96	<3.0
As (µg/g)	53	0.03 ± 0.03	0.02	0.005 - 0.17	<7.0
Cd (µg/g)	45	0.009 ± 0.007	0.007	0.002 - 0.03	<1.0
Pb (µg/g)	53	0.34 ± 0.32	0.25	0.06 - 1.44	<3.0
Water					
Mn (µg/L)	53	0.74 ± 1.66	0.21	0.04 - 10.0	50.0
As (µg/L)	50	0.09 ± 0.05	0.08	0.01 - 0.31	10.0
Cd (µg/L)	27	0.07 ± 0.13	0.03	0.01 - 0.58	3.0
Pb (µg/L)	53	1.43 ± 2.18	0.85	0.02 - 15.0	10.0
Formula					
Mn (µg/L)	16	182 ± 40	180	115 - 243	---

Table 3. Spearman correlations between Mn, Cd, As, and Pb in infant hair (C), mother hair (M), water (W), and formula (F)

	CMn	CAs	CCd	CPb	MMn	MAs	MCd	MPb	WMn	WAs	WCd
CMn	---	---	---	---	---	---	---	---	---	---	---
CAs	0.58***[1]	---	---	---	---	---	---	---	---	---	---
CCd	0.46***	0.45**	---	---	---	---	---	---	---	---	---
CPb	0.27*	0.03	0.54***	---	---	---	---	---	---	---	---
MMn	0.50***	-0.10	0.08	0.22	---	---	---	---	---	---	---
MAs	0.26*	0.15	0.17	0.43***	0.09	---	---	---	---	---	---
MCd	0.20	0.09	0.53***	0.3/**	0.06	0.15	---	---	---	---	---
MPb	-0.08	0.08	0.15	0.31**	0.01	0.28**	0.39***	---	---	---	---
WMn	-0.09	-0.03	0.17	-0.08	0.12	-0.18	-0.01	-0.08	---	---	---
WAs	0.04	0.22	0.20	0.16	-0.08	0.01	-0.04	0.17	0.00	---	---
WCd	-0.17	-0.32	-0.20	-0.10	-0.10	0.27	0.44*	-0.01	0.29	-0.11	---
WPb	-0.03	-0.10	-0.05	-0.06	-0.06	0.07	0.38***	-0.04	0.02	-0.02	0.35*
FMn	-0.18	---	---	---	---	---	---	---	---	---	---

[1] *p<0.1; **p<0.05; ***p<0.01

Table 4. Mn concentration as listed on nutrition labeling in a selected sample of commercial infant formulas

Formula Variety[1]	Mn (µg/5 fl oz)	Mn (µg/L)
Gerber Good Start, Gentle	15	100
Gerber Good Start, Protect	15	100
Gerber Good Start, Soy	25	170
Parent's Choice, Premium	15	100
Parent's Choice, Advantage	5	100
Parent's Choice, Sensitivity	5	30
Parent's Choice, Soy-Based	25	170
Enfamil, Gentlease	15	100
Enfamil, A.R.	15	100
Enfamil, ProSobee	25	170
Similac, Advance	5	30
Similac, Sensitive	5	30
Similac, Soy Isomil	25	170

[1]Soy formulas are specified. Otherwise, milk-based protein used in formulation.

Differences by Feeding Mode

Most demographic variables were not significantly different between formula fed and breastfed infants in this sample (**Table 5**). Mothers who chose to formula feed did not differ from breastfeeding mothers in age, BMI, race, ethnicity, education, employment status, income, or smoking tendencies. Marital status differed significantly between formula feeding and breastfeeding mothers, with 100% of breastfeeding women reporting being married or living with their partner. Of the 4 mothers who reported being single, all (100%) chose to formula feed. There were no differences by feeding mode in whether infants had been introduced to complementary feeding.

Infant growth was also assessed based on feeding mode (**Table 6**). Gestational age or birth measurements were not different between the groups. Current anthropometric measurements were not significantly different based on feeding mode. When calculated as a z-score and percentile taking sex and age into account (based on the WHO method for growth curve z-score calculations, as noted in the methods section), there was a significant difference in both length-for-age z-score and percentile. Formula fed infants were significantly longer than breastfed infants, based both on z-score and percentile ($p = 0.02$ for both). Weight-for-age z-score and percentile were the same between both groups. Weight-for-length z-score and percentile showed significant differences between the feeding modes, with breastfed infants having higher weight compared to their length ($p = 0.04$ for both).

Metal concentrations were assessed for differences between formula fed and breastfed infants (**Table 7**). Infant Mn and As were similar between groups. Cd was significantly different by feeding mode, with breastfed infants having significantly higher hair concentrations ($p = 0.02$). Infants who were breastfed tended to have higher hair Pb concentrations than formula fed infants (7.69 vs. 3.52 μg/g, respectively; $p = 0.07$). Mothers had similar hair concentrations of all metals except As when compared based on how their infants were fed. As was significantly

higher in women who formula fed their infants ($p = 0.04$). Water concentrations of Mn, Cd, and As were similar for samples from breastfed and formula fed infants. A significant difference was seen with water Pb concentrations; higher concentrations were seen in water samples from breastfed infants than from formula fed infants (1.77 vs. 0.82 µg/L, respectively; $p = 0.003$).

Table 5. Differences in demographic and maternal variables by feeding mode

	Mean ± SD or n(%)		*p*-value[1,2]
	Breastfed n = 34	Formula Fed n = 19	
Maternal Age (years)	30.96 ± 0.63	30.54 ± 1.24	0.42
Pre-pregnancy BMI (kg/m^2)[3]	25.96 ± 1.38	26.80 ± 1.62	0.48
Current Complementary Feeding			0.42
No	20 (69.0)	9 (31.0)	
Yes	14 (58.3)	10 (41.7)	
Age of Complementary Food Introduction	19.2 ± 3.6	15.2 ± 8.4	0.23
Maternal Smoking in Pregnancy			0.99
No	33 (64.7)	18 (35.3)	
Yes	1 (50.0)	1 (50.0)	
Maternal Current Smoking			0.29
No	33 (44.0)	17 (34.0)	
Yes	1 (33.3)	2 (66.7)	
Mother Ethnicity			0.99
Non-Hispanic	33 (64.7)	18 (35.3)	
Hispanic	1 (50.0)	1 (50.0)	
Child Ethnicity			0.12
Non-Hispanic	34 (66.7)	17 (33.3)	
Hispanic	0 (0.0)	2 (100.0)	
Mother Race			0.99
Caucasian	33 (63.5)	19 (36.5)	
Other	1 (100.0)	0 (0.0)	
Child Race			0.53
Caucasian	32 (62.8)	19 (37.2)	
Other	2 (100.0)	0 (0.0)	
Income			0.44
<$19,999	1 (33.3)	2 (66.7)	
$20,000-39,999	7 (70.0)	3 (30.0)	
$40,000-59,999	10 (58.8)	7 (41.2)	
$60,000-79,999	8 (80.0)	2 (20.0)	
$80,000-99,999	5 (50.0)	5 (50.0)	
>$100,000	3 (100.0)	0 (0.0)	
Marital Status			0.01
Married or Living with Partner	34 (69.4)	15 (30.6)	
Single	0 (0.0)	4 (100.0)	
Maternal Education			0.22
Some College	3 (37.5)	5 (62.5)	
College	16 (66.7)	8 (33.3)	
Post-Graduate	15 (71.4)	6 (28.6)	
Maternal Occupation			0.34
Employed	22 (57.9)	16 (42.1)	
Maternity Leave	2 (100.0)	0 (0.0)	
Unemployed	10 (76.9)	3 (23.1)	

[1]*p*-values for n(%) calculated by chi-squared test where cell sites include >5 participants, or Fisher's exact test where cell sites include ≤5 participants; [2]*p*-values for mean ± SD calculated using Wilcoxon Rank Sum test; [3]Pre-pregnancy BMI calculated based on self-reported height and pre-pregnancy weight.

Table 6. Differences in child growth variables by feeding mode

	Mean ± SD or n(%)		*p*-value
	Breastfed	Formula Fed	
Age (months)	4.74 ± 0.21	4.44 ± 0.28	0.14
Sex			0.57
Male	13 (59.1)	9 (40.9)	
Female	21 (67.7)	10 (32.3)	
Gestational Age (weeks)	39.5 ± 0.23	39.5 ± 0.34	0.46
Birth Anthropometric Data			
Birth Length (cm)	52.2 ± 0.55	52.8 ± 0.75	0.19
Birth Weight (g)	3398.8 ± 86.9	3468.5 ± 163.8	0.37
Current Anthropometric Data			
Current Length (cm)	63.8 ± 0.65	64.7 ± 0.74	0.18
Current Weight (g)	6729.9 ± 179.2	6772.2 ± 234.6	0.44
Length for Age Z-score	0.047 ± 0.18	0.639 ± 0.13	0.02
Length Percentile	50.67 ± 5.46	71.48 ± 4.15	0.02
Weight for Age Z-score	-0.070 ± 0.155	0.125 ± 0.224	0.43
Weight Percentile	47.17 ± 4.75	51.50 ± 6.66	0.43
Weight for Length Z-Score	-0.097 ± 0.156	-0.606 ± 0.271	0.04
Weight for Length Percentile	46.39 ± 4.50	33.40 ± 7.64	0.04

Table 7. Differences in metal concentrations by feeding mode[1]

	Mean ± SD or n(%)		*p*-value
	Breastfed	Formula Fed	
Infant Hair Metal Concentrations			
Mn (µg/g)	2.21 ± 0.46	2.01 ± 0.67	0.11
Cd (µg/g)	0.62 ± 0.27	0.16 ± 0.05	0.02
As (µg/g)	0.18 ± 0.07	0.27 ± 0.10	0.28
Pb (µg/g)	7.69 ± 2.05	3.52 ± 0.85	0.07
Mother Hair Metal Concentrations			
Mn (µg/g)	0.27 ± 0.11	0.21 ± 0.31	0.44
Cd (µg/g)	0.010 ± 0.001	0.008 ± 0.002	0.12
As (µg/g)	0.027 ± 0.002	0.031 ± 0.009	0.04
Pb (µg/g)	0.31 ± 0.05	0.40 ± 0.09	0.36
Water Metal Concentrations			
Mn (µg/L)	0.83 ± 0.34	0.58 ± 0.19	0.25
Cd (µg/L)	0.053 ± 0.024	0.061 ± 0.044	0.19
As (µg/L)	0.084 ± 0.007	0.109 ± 0.016	0.13
Pb (µg/L)	1.77 ± 0.44	0.82 ± 0.25	0.003

[1]Hair Mn, As, Cd, Pb concentrations log-transformed for this analysis.

Predictors of Maternal Hair Mn Concentrations

Bivariate regression models were used to investigate predictors of maternal hair Mn concentrations (**Table 8**). Maternal education and employment status were significantly negatively associated with hair Mn concentration. No other demographic variables were significant in bivariate regression. Hair metal concentrations were not associated with maternal Mn concentration, nor were water As, Cd, and Pb. Water Mn concentration was strongly positively associated with maternal hair Mn ($p = 0.003$). Maternal education and employment, and water Mn concentration remained significant predictors of maternal hair Mn concentration in multivariate modeling (**Table 9**).

Table 8. Bivariate predictors of maternal hair Mn concentration (μg/g)[1]

	n	β ± SE	*p*-value
Smoking During Pregnancy			
No	51	REF	---
Yes	2	0.65 ± 0.58	0.27
Current Smoking			
No	50	REF	---
Yes	3	0.17 ± 0.58	0.72
Maternal Ethnicity			
Non-Hispanic	51	REF	---
Hispanic	2	0.21 ± 0.59	0.72
Maternal Race			
Caucasian	52	REF	---
Other[2]	1	0.37 ± 0.82	0.65
Income			
>$100,000	3	REF	---
$80,000-99,999	10	0.68 ± 0.54	0.21
$60,000-79,999	10	0.17 ± 0.51	0.74
$40,000-59,999	17	0.23 ± 0.54	0.67
$20,000-39,999	10	0.12 ± 0.54	0.83
<$19,999	3	-0.13 ± 0.66	0.85
Marital Status			
Married or Living with Partner	49	REF	---
Single	4	0.29 ± 0.42	0.5
Education			
Some College or Below	11	REF	---
College	24	-0.30 ± 0.33	0.36
Post-Graduate	18	-0.65 ± 0.33	0.05
Employment Status			
Unemployed	13	REF	---
Maternity Leave	2	0.20 ± 0.59	0.74
Employed	38	0.60 ± 0.25	0.02
BMI (kg/m^2)[3]	52	0.08 ± 0.13	0.53
Hair Metal Concentrations			
As (μg/g)	52	0.0413 ± 0.156	0.79
Cd (μg/g)	44	0.006 ± 0.186	0.98
Pb (μg/g)	52	0.107 ± 0.134	0.43
Water Metal Concentrations			
Mn (μg/L)	52	0.229 ± 0.060	0.003
As (μg/L)	49	0.379 ± 2.311	0.87
Cd (μg/L)	26	0.133 ± 1.112	0.91
Pb (μg/L)	52	0.039 ± 0.051	0.45

[1]Hair Mn, As, Cd, Pb values log-transformed for this analysis; [2]Other includes non-Hispanic Black and Asian; [3]Pre-pregnancy BMI based on self-reported height and pre-pregnancy weight.

Table 9. Multivariate predictors of maternal hair Mn concentration (μg/g)[1]

	β ± SE	*p*-value
Intercept	-2.17 ± 0.15	<0.01
Water Mn Concentration (μg/L)	0.20 ± 0.06	<0.01
Education		
Some College or Below	REF	---
College	-0.32 ± 0.28	0.27
Post-Graduate	-0.60 ± 0.28	0.04
Employment Status		
Unemployed	REF	---
Maternity Leave	0.19 ± 0.52	0.72
Employed	0.51 ± 0.22	0.02

[1]Hair Mn log-transformed for this analysis.

Predictors of Infant Hair Mn Concentrations

Potential covariates were first tested in a bivariate model to assess if each was a salient predictor of infant hair Mn concentration (**Table 10**). No current complementary feeding tended to be negatively associated with infant hair Mn. Maternal current smoking was significantly positively associated with Mn concentration. Maternal education was negatively associated with hair Mn, so that with increasing education status, hair Mn decreased. Infant hair Mn was positively associated with both weight-for-length z-score and percentile ($p = 0.02$ for both). Strong associations were seen with other infant hair metals and maternal hair metals. Infant hair As increased along with hair Mn ($\beta = 0.55$, $p < 0.001$). Infant hair Cd was also positively associated with infant hair Mn ($\beta = 0.39$, $p < 0.001$). Pb also strongly predicted Mn ($\beta = 0.37$, $p < 0.001$). Maternal hair Mn and As were strong positive predictors of infant hair Mn concentrations (Mn: $\beta = 0.41$, $p = 0.01$; As: $\beta = 0.39$, $p = 0.03$).

Based on bivariate associations between potential covariates and infant hair Mn concentration, a multivariate model was constructed (**Table 11**). The full model included variables that were associated with infant hair Mn at a significance of $p < 0.15$. Since both weight-for-length z-score and percentile were significantly and positively associated with infant Mn status but were highly correlated, (Pearson's $r = 0.98$, $p < 0.001$) z-score was chosen to be included in the model. A sensitivity analysis confirmed similar results obtained when weight-for-length percentile was used in the model (Appendix C). Variables included in multivariate model 1 were infant hair As, Cd, and Pb, mother hair Mn and As, mother current smoking habits, and weight-for-length z-score, with infant hair Mn as the dependent variable. This model explained 69.44% of variability in the data (Adjusted $R^2 = 0.6231$). The full model confirmed that infant hair As and mother hair Mn concentrations were strong predictors of infant hair Mn concentration (Infant As: $\beta = 0.43$, $p < 0.001$; Mother Mn: $\beta = 0.25$, $p = 0.05$). Weight-for-length z-score maintained a trend for significance in the full model ($\beta = 0.21$, $p = 0.06$).

Table 10. Bivariate predictors of infant hair Mn concentration (μg/g)[1]

	n	β ± SE	*p*-value
Age (months)	51	0.01 ± 0.11	0.98
Primary Feeding Mode			
Breastfed	34	REF	---
Formula Fed	19	0.33 ± 0.27	0.22
Complementary Feeding Introduced			
Yes	24	REF	---
No	29	-0.45 ± 0.25	0.09
Maternal Age	51	-0.01 ± 0.03	0.74
Maternal Smoking During Pregnancy			
No	51	REF	---
Yes	2	1.09 ± 0.65	0.10
Maternal Current Smoking			
No	3	REF	---
Yes	50	1.10 ± 0.53	0.04
Child Ethnicity			
Non-Hispanic	51	REF	---
Hispanic	2	-1.02 ± 0.66	0.13
Child Race			
Caucasian	51	REF	---
Other[2]	2	0.35 ± 0.67	0.61
Income			
>$100,000	3	REF	---
$80,000-99,999	10	0.78 ± 0.60	0.20
$60,000-79,999	10	0.39 ± 0.57	0.50
$40,000-59,999	17	0.22 ± 0.60	0.71
$20,000-39,999	10	-0.14 ± 0.60	0.82
<$19,999	3	-0.18 ± 0.74	0.81
Maternal Education			
Some College or Below	11	REF	---
College	24	-0.77 ± 0.39	0.05
Post-Graduate	18	-0.75 ± 0.40	0.07
Employed			
Unemployed	13	REF	---
Maternity Leave	2	1.02 ± 0.70	0.15
Employed	38	0.42 ± 0.30	0.18
Maternal BMI (kg/m^2)[3]	50	0.01 ± 0.17	0.72

(Table 10 continued)

	n	β ± SE	*p*-value
Anthropometric Variables			
Current Weight	51	0.0001 ± 0.0001	0.27
Current Length	50	-0.02 ± 0.04	0.66
Length for Age Z-score	50	-0.17 ± 0.15	0.25
Length Percentile	50	-0.006 ± 0.005	0.19
Weight for Age Z-score	51	0.13 ± 0.14	0.37
Weight Percentile	51	0.003 ± 0.005	0.52
Weight for Length Z-score	50	0.29 ± 0.12	0.02
Weight for Length Percentile	50	0.01 ± 0.01	0.02
Infant Hair Metal Concentrations			
As (µg/g)	46	0.55 ± 0.08	<0.001
Cd (µg/g)	43	0.39 ± 0.09	<0.001
Pb (µg/g)	51	0.37 ± 0.10	<0.001
Mother Hair Metal Concentrations			
Mn (µg/g)	50	0.41 ± 0.15	0.01
As (µg/g)	50	0.39 ± 0.17	0.03
Cd (µg/g)	43	0.26 ± 0.20	0.20
Pb (µg/g)	50	-0.03 ± 0.16	0.83
Water Metal Concentrations			
Mn (µg/L)	51	-0.004 ± 0.078	0.96
As (µg/L)	48	0.63 ± 2.46	0.80
Cd (µg/L)	26	-1.76 ± 1.36	0.21
Pb (µg/L)	51	-0.03 ± 0.06	0.64
Infant Formula Concentration	16	-5.11 ± 6.79	0.46

[1]Hair Mn, As, Cd, Pb values log-transformed for this analysis; [2]Other includes non-Hispanic Black and Asian; [3]Pre-pregnancy BMI based on self-reported height and pre-pregnancy weight.

Table 11. Multivariate predictors of infant hair Mn concentrations (µg/g)[1]

	β ± SE	*p*-value
Intercept	2.43 ± 1.48	0.11
Infant Hair Metal Concentrations		
As (µg/g)	0.43 ± 0.10	<0.001
Cd (µg/g)	0.03 ± 0.13	0.85
Pb (µg/g)	0.12 ± 0.15	0.42
Mother Hair Metal Concentrations		
Mn (µg/g)	0.25 ± 0.12	0.05
As (µg/g)	-0.03 ± 0.20	0.89
Complementary Feeding Introduced		
Yes	REF	---
No	-0.17 ± 0.20	0.40
Maternal Smoking During Pregnancy		
No	REF	---
Yes	0.07 ± 0.57	0.90
Maternal Current Smoking		
No	REF	---
Yes	0.38 ± 0.42	0.37
Child Ethnicity		
Non-Hispanic	REF	---
Hispanic	-0.19 ± 0.64	0.77
Maternal Education		
Some College or Below	REF	
College	-0.37 ± 0.34	0.30
Post-Graduate	-0.46 ± 0.33	0.17
Weight-for-Length Z-Score	0.21 ± 0.11	0.06

[1]Hair metal levels log-transformed for this analysis.

Chapter 4

Discussion

Infant formula contains a high concentration of Mn, and may serve as a potential exposure source of the metal during infancy. Prior studies have found cognitive and behavioral deficits with environmental exposure to Mn, but dietary exposure has been rarely studied. This study investigated infant hair Mn concentrations based on feeding mode, and determined other important predictors of Mn in infant hair.

In this study of hair Mn concentrations in breastfed and formula fed infants, Mn was not different based on feeding mode. However, infants in this study who were breastfed had higher hair Cd and Pb, and were shorter in length than formula fed infants. Mothers of breastfed infants were more likely to be married or living with their partner and had lower hair As concentrations. Tap water of breastfed infants was lower in As and higher in Pb compared to tap water from the homes of formula fed infants. The factors that predicted infant hair Mn in multivariate models included infant hair As, maternal hair Mn, and weight-for-length.

Hair, Water, Formula Metal Levels

Metal concentrations found in the hair and water of this sample were not indicative of high exposures of Mn, As, and Cd. Infant hair Mn concentrations averaged within the normal range for hair Mn according to Miekeley and colleagues (1998), who proposed 3 μg/g to be the cutoff for elevated hair Mn. This value has been well accepted and used in various other studies (Wasserman 2006, 2011). Of all infants in this study, seven had hair Mn concentrations above the cutoff, and thus could be considered to have elevated concentrations. Five of these infants were breastfed, and two were formula fed. The mean hair Mn concentration seen in this study

was below concentrations seen in one study of children exposed to Mn through well water (6.6 µg/g, Bouchard 2007), but higher than concentrations seen in another study looking at children with elevated tap water Mn (1.1 µg/g, Bouchard 2010). These studies noted increased hyperactive and oppositional behaviors and decreased IQ scores associated with hair Mn. Children living in a mining area had a hair concentration of 12.6 µg/g when exposed to very high metal levels in the local surroundings, compared to 0.6 µg/g in controls (Riojas-Rodríguez 2010). A study of cognition and metal exposure in school aged children in Spain did not find any associations between attention, visuospatial capabilities, or abstract reasoning and hair Mn at levels of 0.18 µg/g in an industrial area (Torrente 2005). Another study of learning disabilities found that a mean Mn hair concentration was significantly higher in learning disabled children compared to controls (0.83 vs. 0.58 µg/g, respectively) (Pihl 1977). Hair Mn levels seen in the present investigation surpass levels in all but two of these studies, though remain below the proposed elevated cutoff level. Due to the nature of the study, it is unknown whether hair Mn levels seen here might be associated with measures of behavioral or cognitive function. Interestingly, the studies finding lower hair Mn concentrations than the present investigation showed significant associations with cognitive deficits and behavioral problems.

While As and Cd were low in hair concentrations for both mothers and infants in this sample, Pb was elevated in infant hair, with a mean of 6.22 µg Pb/g compared to a proposed cutoff of 3 µg/g for normal levels (Park 2006). Children living in an industrial complex showed associations between cognition and Pb exposure at hair levels of 1.59 µg/g, much lower than levels seen in the present study (Torrente 2005). In contrast, a study of children living in an area of Poland that is very highly contaminated with heavy metals due to Zn industrial plants showed children in the area had a mean hair Pb of 8.21 µg/g (Chlopicka 1998), showing higher hair Pb concentrations in children with industrial exposure than the infants in the present study. Blood has been considered the standard measurement for accurate assessment of Pb exposure, though

hair has been suggested to be a useful screening alternative (Wibowo 2004). Based on previous studies, the hair Pb levels seen in this study are in fact elevated.

Water metal concentrations from tap water samples in this area did not indicate high Mn exposure, as the mean was well below the EPA suggested maximum limit of 50 µg/L (EPA 2004). There have not been reports of high Mn in State College and the surrounding areas in Pennsylvania, so the detected levels were expected. Pb concentrations seen in tap water samples were low as well, with the mean and median falling below the WHO recommended upper limit of 10 µg/L (WHO 2008). This is indicative that the sample analyzed in this study was not lead exposed from water.

Infant formulas have been reported in the past to range from 50-300.0 µg/L in Mn content (Lönnerdal 1994). Current commercial infant formulas report a range of 30-170 µg Mn/L based on nutrition labeling, with the highest reported content coming from soy formulas. Most formula samples analyzed in this study fell within this range, with a mean content of 182 µg Mn/L, though some samples contained as much as 243 µg Mn/L. With breast milk ranging from 3-8 µg/L (Lönnerdal 1994), the formula samples in this study reached as much as 81 times the concentration of Mn found in human breast milk. All infant formulas reported by participants in the current study listed a Mn concentration of 100 µg/L (15 µg per fluid ounce) on the package nutrition labeling, which was exceeded by every sample in atomic absorption spectrometry analysis. While Mn concentrations fell within ranges previously reported for scientific analysis of Mn in infant formula, it is particularly interesting that all samples exceeded company reported concentrations by as much as 143 µg/L. This could be for several reasons. First, the infant formula could indeed contain more Mn than reported on nutrition labels. Second, mothers could be mixing a higher formula powder or concentrate to water ratio when making up the formula, so that there is a higher concentration of energy and nutrients per fluid ounce than listed based on suggested mixing proportions. Third, there could be an instrument bias, skewing our results. The

instrument was carefully calibrated to a standard curve using a professionally mixed standard for Mn developed for atomic absorption spectrometers specifically, and samples were read using a Mn-specific element lamp. With this in mind, it is unlikely that the instrument would have a big enough bias to impact the sample readings significantly. It is most likely that there is a higher Mn concentration in the formula due to improper mixing of the formula by mothers because of inaccurate measuring of concentrated formula.

Differences by Feeding Mode

Demographic Variables

Most mothers in the present sample reported at least a college education and income of greater than $40,000. The sample was mostly non-Hispanic Caucasian, and was fairly homogeneous in race and ethnicity. There were few demographic differences between feeding mode groups. A greater proportion of mothers who breastfed were married (100% vs. 78.9% in formula feeding mothers). This is consistent with previous reports of demographic predictors of breastfeeding (Lande 2003). Race, ethnicity, education, and income are also well-known predictors of breastfeeding, though we did not see significant differences between feeding groups in this study (Lande 2003). The lack of variability of the study sample in these variables limited any ability to determine differences in these factors by infant feeding mode.

Anthropometric Variables

Infants who were breastfed or formula fed had similar birth weight, birth length, and gestational age. The lack of differences in these variables strengthened the anthropometric findings at the time of data collection. Infants who were formula fed tended to be longer (71.5^{th} vs. 50.7^{th} percentile length-for age), even with gender and age taken into account per the WHO

growth z-score and percentile calculations. Weight-for-length did differ between groups, and was driven by length as there were no significant differences in weight. This finding is supported by previous studies of growth and infant feeding. One study found infants who were breastfed tended to gain in both length and weight more slowly than formula fed infants through 31 months of age (Ong 2002). A review of 19 studies of infant growth concluded that breastfed infants tend to be lower in weight-for-length and have slower length gain than formula fed infants (Dewey 1998), which is consistent with the current findings. It was surprising to see no differences in weight between breastfed and formula fed infants, as many studies have found breastfed infants to gain less weight through the first year of life (Dewey 1998, Dewey 1992, Ogden 2002).

Infant Hair Metal Concentrations

One of the main aims of this study was to determine whether infants who were formula fed and infants who were breastfed had different concentrations of hair Mn as a marker of Mn status. Based on the analysis done on hair samples collected from infants in each feeding group, no differences were found in Mn concentration. Additionally, neither group exhibited mean hair Mn concentrations in excess of proposed normal ranges. This indicates that infant formula did not result in high body Mn burdens. The sample size of this study was small and groups were of uneven sizes, which could contribute to the lack of effect noted. To account for uneven group sizes, nonparametric statistics were used; even so, no differences were seen. It is possible that the total power for the study was not adequate to see differences. However, a highly significant difference was seen between length-for-age and weight-for-length z-scores and percentiles for each group, indicating that there was enough power to detect group differences. A *post hoc* power calculation indicated that at least 45 infants per feeding group would be required to see differences at the metal levels seen in this analysis, though the present study parameters were at a 70% power level.

While few studies have looked at infant formula as a dietary source of Mn, similar results have been previously reported. Stastny and colleagues found no differences in serum Mn between infants who were formula fed and infants who were breastfed (1984). This study found Mn intake in breastfed infants to be significantly correlated with serum levels, but found no correlation between intake and serum Mn in formula fed infants. The authors posit the difference in correlations is due to decreased bioavailability of Mn from infant formula due to phytates and high concentrations of micronutrients that decrease Mn absorption (iron, calcium, and phosphorous in particular). One other study did look at longitudinal differences in hair Mn concentrations, comparing concentrations at birth to 6 weeks, 4 months, 9 months, and 3 years of age in formula fed children (Collipp 1983). Hair Mn concentrations varied across the time span, increasing at 6 weeks, then decreasing continuously for all time points. Infants 4 months of age were compared by feeding mode, finding significantly higher hair Mn in formula fed infants compared to breastfed infants. However, there was a very limited sample size (10 infants in each group), and it was not specified if infants were exclusively fed one feeding mode or the other. Additionally, soy or cow's milk based infant formulas were not specified for the analysis, and no infant formula samples were tested for verification of Mn content. Infant formulas also were much higher in Mn content at the time of the study compared to now; Mn content in formula has been reduced since Collipp's study was published.

The results of the present study indicate that formula fed infants do not exhibit higher Mn concentrations than breastfed infants, despite the high amount of the metal found in infant formula. Infant formula contains high amounts of phytates, which have been shown to limit the absorption of Mn in the gut (Davidsson 1991). Additionally, infant formula is typically high in iron, calcium, and zinc, micronutrients that have also been shown to decrease Mn absorption (Davidsson 1991, Planells 2000). Thus, infants consuming formula may not be taking up the full amount of Mn provided from the formula due to these inhibitory compounds. Taking into

consideration the finding that there were no differences in hair Mn by feeding mode and keeping in mind previous findings of neurotoxicity in children exposed environmentally, the results of this study suggest that source of exposure to Mn may determine whether toxicity is experienced, with environmental exposure being more detrimental. This may be partly explained by the fact that dietary Mn plays an important nutritive role in contributing adequately to physiological requirements (IOM 2001).

Infants did differ significantly in Cd concentrations, with breastfed infants having higher hair concentrations. Similarly, a trend was seen for infants who were breastfed to have higher Pb concentrations than formula fed infants. Mothers of breastfed infants did not have higher Pb concentrations in hair than formula fed infants, though tap water of breastfed infants was significantly higher in Pb. Lactation has been shown to mobilize Pb stores from bone, where over 90% of the metal is stored in the adult body (Téllez-Rojo 2002, Barry 1970, Barry 1975). Pb exposure in breastfed infants is strongly affected by maternal bone stores and previous exposure, along with diet and nutritional factors (current Pb exposure, factors that enhance or inhibit Pb absorption) (Dorea 2006). In this study, water Pb concentrations were significantly higher in samples from breastfed infants and their mothers, although surprisingly these mothers did not have higher hair Pb concentrations than mothers of formula fed infants. Infants who are formula fed may be exposed to Pb through tap water used in reconstitution of the formula (Baum 1997). The water Pb concentrations in formula fed infants was quite low, indicating this group experiences low Pb exposure from dietary sources (as the water is mixed with concentrated formula powder to reconstitute the formula). These factors explain higher Pb seen in breastfed infants. Infant hair Pb in this sample showed a trend for an association with household income ($p = 0.06$), further strengthening the fact that Pb exposure in this population is behaving consistently with previous research.

Predictors of Maternal Hair Mn Concentration

Maternal Mn in hair samples was strongly and positively predicted by concentrations of Mn in tap water samples. This relationship has been seen and well documented in studies of environmental exposure to metals. Water has been shown to be a significant source of ingested Mn. Though water levels were low in this sample, it seems that it is still an important exposure source for adults. A study of Mn exposure in Quebec found an association between hair and water Mn concentrations, in line with the current findings (Bouchard 2011).

Maternal hair Mn was negatively associated with education, suggesting that with increasing education status (from some college or below up to post-graduate education) there was decreasing hair Mn. Previous studies have shown similar results for Mn exposure. An investigation of risk of toxic metal exposure in central Mexico found an inverse significant association between blood Mn concentrations and education, though this relationship remained unexplained (Santos-Burgoa 2001). Few other studies have investigated demographic correlates of Mn exposure in adults. Most have looked at neuropsychological variables associated with exposure markers, and thus have automatically included education as a covariate without reporting potential predictive value for Mn status. This finding follows previous studies that have reported a negative association between toxic metal exposures and socioeconomic status (Berglund 2011).

Employment status was predictive of hair Mn, with women who were employed or a full-time student (with a provided stipend) having lower Mn concentrations in bivariate regression models than women who were unemployed or on maternity leave. It is possible that employment served as a potential route of exposure in this sample, as employed mothers or mothers on maternity leave showed a positive association with hair Mn. It is possible that water consumed at work of ambient exposure near the workplace are higher in Mn than the places of residence near State College, resulting in higher Mn burdens in these women.

Predictors of Infant Hair Mn

In bivariate regression modeling, several potential predictors of infant hair Mn were identified. Infants who were consuming complementary foods were more likely to have higher hair Mn. Complementary foods introduced included fruits, vegetables, and grains (rice and oat based cereals). These food items tend to be good sources of Mn. Infants consuming these additional foods would be ingesting more sources of Mn, potentially adding to the body Mn load. However, it is likely that the quantity of complementary foods being consumed by the infants is quite small, and has minimal impact on infant body burden of Mn. This is further supported by the lack of significance of this variable with hair Mn in the multivariate model.

Infant As, Cd, and Pb were strong predictors of hair Mn concentration in bivariate models. Previous studies have found correlations between these metals, particularly between Mn, Cd, and As (Wright 2006). A study of multiple metal exposures found Pb, Mn, Cd, and As to all be at least moderately correlated with one another (Kordas 2010). Strong associations between hair metals contribute further evidence that risk of multi-metal exposures may be common, and warrant further research.

In the full models, infant hair As, mother hair Mn, and infant weight-for-length were salient predictors of infant hair Mn. Several previous studies in Bangladesh have looked at the environmental co-exposure between Mn and As in children. Water Mn and As were strongly correlated in these studies, and both were associated with decreasing IQ scores in unadjusted models (Wasserman 2004). However, when exposure to Mn was adjusted for As, no effects remained. In contrast, when Mn was included in the model for As association with IQ, a significant effect was still seen. In another study from the same group, when exposure was categorized as high or low for both As and Mn, both metals exhibited significant negative effects on IQ, even when adjusted for the other metal in addition to relevant covariates (Wasserman 2011). In a younger group of children, As maintained a strong negative effect on IQ, which

remained with covariate adjustment (Wasserman 2007). It seems from these studies that Mn and As are correlated and co-exposure may be of particular concern. However, in assessing intellectual function, As may be driving the detrimental effects due to metal exposure. The results found in the present study further strengthen the relationship between Mn and As exposure in children, suggesting they may be an important co-exposure.

Maternal Mn concentration remained a strong predictor in multivariate modeling. This result was expected, based on previous studies showing that maternal hair Mn is significantly correlated to children's hair Mn, and hair Mn tends to be strongly associated within families (Menezes-Filho 2011, Haynes 2010, Kordas 2010). As ingested exposure did not seem to impact infant hair Mn, evidenced by the lack of difference in hair Mn between formula fed and breastfed infants, it is likely that any Mn exposure sources for infants are environmental and are also experienced by the mother.

Weight-for-length z-score was a strong predictor of Mn concentration in bivariate regression, and remained a marginally significant predictor of infant hair Mn concentration in the full predictive models. With increasing growth percentile, infant hair Mn tended to increase as well. While other studies have noted associations between Mn and weight status, few have investigated length or overall growth in relation to exposure. Infant birth anthropometric data was investigated in a previous study looking at maternal blood Mn and birth weight (Zota 2009). Maternal Mn levels in blood at delivery were found to have a nonlinear association with birth weight, with birth weight decreasing at low and high maternal blood Mn concentrations. This study did not investigate length at birth. In a study of metal exposures, Mexican children exposed to Mn due to proximity to a mining district had a higher prevalence of stunting compared to non-exposed controls, though the difference was not statistically significant (Hernandez-Bonilla 2011). A potential explanation for the positive association found in this study is that Mn is acting as a nutrient. Mn plays an important role in growth; it is a necessary component of enzymes

including glutamate synthase which is essential for glutamine synthesis and glycosyltransferases and xylosyltransferases which are important for proteoglycan synthesis and bone formation (IOM 2001). Children who have a higher weight-for-length z-score may be requiring greater amounts of Mn to support such growth, and are absorbing more of the nutrient in response to biological need. Further research should be conducted to better investigate the associations between Mn and growth status in infants.

Strengths and Limitations

There are some limitations that must be considered in this study. The sample size of the current analysis is quite small and sample size is biased towards breastfed infants (n=34 breastfed infants, n=19 formula fed infants). A small sample size may limit detection of the effects of dietary intake on outcomes of interest. However, the number of participants in the study was adequate to identify significant differences in some hair metal, anthropometric, and demographic variables, showing that there was enough power to see significant effects.

We did not assess the dietary Mn contributions of complementary feeding in this study. Many infants (45.3%) in the study had been introduced to other foods beyond breast milk and infant formula. While we were not able to assess other Mn sources, all infants in the study were receiving at least 70% of the diet from breast milk or formula, as it was an eligibility criterion. In addition, the quantity of infant formula consumed daily by formula fed infants was not collected in this study, so the total daily Mn intake was not available for analysis. Due to the scope of this research and logistical limitations, breast milk samples were not collected which limits assessment of Mn intake for breastfed infants. Formula samples were assessed for Mn concentration, which was important for determining correlations between Mn concentrations in formula and infant hair. Future studies should do additional dietary probing to determine quantity

of Mn consumed by infants of each feeding mode in order to relate those intake concentrations to markers of Mn status, and potentially to developmental outcomes.

Hair was the only biomarker used to assess body Mn status. Blood was not used in this study to minimize invasiveness of protocol and to minimize costs of sample collection and analysis. Hair has been well accepted as a measure of bodily Mn (Creason 1975, Guillard 1984). Several previous studies of Mn exposure during childhood have used both hair and blood measures together and have found strong correlations between the two measures (Hernandez-Bonilla 2011, Wasserman 2011). Another study of seasonal trace element intakes found significant associations between ingested Mn from dietary sources and hair Mn concentrations (Ross 1986).

While there were some limitations, this study had several important strengths. We were able to collect information on many covariates to assess their potential predictive ability of infant and maternal Mn levels. Anthropometric measurements taken in the laboratory were calculated as z-scores and percentiles based on WHO recommendations to a sense of growth by age and sex. Demographic variables were well described in the questionnaire, with all mothers willing to provide full information. This study also analyzed samples of infant formulas to determine the actual Mn concentration as prepared, rather than relying on nutrition labels. The one previous study on infant formula Mn in humans limited infant formulas in the study, and did not adequately determine actual contribution of the formulas to Mn in the diet (Collip 1983). The laboratory analysis of infant formula in the present investigation strengthened our ability to detect whether infant formula predicts infant hair Mn in formula fed children.

Chapter 5

Conclusion

The present study found no significant differences in hair Mn concentration between formula fed and breastfed infants. Previous studies have shown drastic differences in Mn provided by feeding mode, with formula providing as much as 50-80 times the amount of the nutritive metal compared to breast milk (Lönnerdal 1994). Our study confirmed the high concentration of Mn contained in various non-soy infant formulas. There was no significant difference in hair Mn between feeding groups despite finding high Mn in formula. Though no differences were seen between feeding groups, it is important for infant formula companies to reconsider the quantity of Mn in product formulations. More studies of Mn bioavailability and absorption in human infants are necessary to best understand how much Mn is optimal in infant formulas. In addition, infant formula Mn concentration was not predictive of infant hair Mn for formula fed infants. These findings suggest that ingestion may not be an important exposure mechanism for Mn toxicity in infants. It is possible that Mn from infant formula is less bioavailable, explaining the lack of difference. One implication of these findings is that studies of dietary Mn exposure from formula in settings where environmental sources are prevalent may not be able to separate effects of environmental from ingested Mn on body Mn burdens or functional outcomes.

No studies have investigated salient predictors of hair Mn in infants below 6 months of age. This study provides an important contribution to toxicological and nutrition studies with findings of important variables that predict biomarkers of Mn exposure in infants. The importance of environmental sources rather than ingested sources of Mn exposure was further supported in the present study by a strong positive association between maternal and infant hair Mn, regardless of infant feeding mode. This finding may have important implications on future

studies of neuropsychological effects of Mn toxicity, as there may be a two-fold effect to consider: both the impact of Mn toxicity directly on the child, but also the effect of Mn toxicity on caregivers, which may impact the development of the child. Socioeconomic status was found to be inversely associated with maternal Mn, as expected based on previous studies of metal exposures. Based on this finding, it is particularly important to focus on low socioeconomic status populations when screening for Mn exposure, as there may be a particularly high risk for toxicity.

The present study has looked at exposure to ingested Mn in infants. Future studies should expand upon the present findings. A study with a larger sample size that additionally explores soy-based infant formulas would bring additional confidence to the results of this work.

This study found hair As to be predictive of hair Mn in infants. While some studies have looked at the interactive effects of these two elements on cognition, the prevalence of co-exposure is not well known in this region. Future studies should be aware of the predictive capacity of As for Mn concentrations, and may find it beneficial to investigate potential interactive effects of concurrent exposure on functional outcomes.

Infant growth was predictive of Mn status in the studied population, an association that has not been previously reported. Future investigations should look into the role of Mn, both acting as a toxicant and as a nutrient, on growth during infancy and childhood. This will be particularly important to better understand the underlying mechanisms for this association.

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Appendix A

Participant Feeding Questionnaire

Subject ID: __ __ __ __
Date: __ __ / __ __/ __ __

1. Is your baby currently taking any medications or supplements (vitamins, minerals, herbs, etc.)? □ Yes (specify):_____
□ No

2. Has your baby's doctor told you that your baby has any of the following?

Poor weight gain	□ Yes	□ No
Gastroesophageal Reflux Disease (GERD) requiring medication	□ Yes	□ No
Allergy or intolerance to breast milk	□ Yes	□ No
Allergy or intolerance to formula	□ Yes	□ No
Blood in stool	□ Yes	□ No
Dehydration	□ Yes	□ No

3. If you can, please tell me what percentage of your infant's diet was made up of either breastmilk or formula (whichever is easier for you) for each month since birth. Let's begin with month 1 after birth:

Age	% Breastmilk	% Formula
Month 1		
Month 2		
Month 3		
Month 4		
Month 5		
Month 6		

Now let's review your infant's diet on a weekly basis since 3 months of age. If you can, please tell me what percentage of your infant's diet was made up of breastmilk or formula at:

Age	% Breastmilk	% Formula
Week 13		
Week 14		
Week 15		
Week 16		
Week 17		
Week 18		
Week 19		
Week 20		
Week 21		
Week 22		
Week 23		
Week 24		

4. What are you **currently** feeding your baby?
□ Breast milk only →Go to Question 8
□ Both breast milk and formula →Go to Question 5
□ Formula only →Go to Question 6

5. What percentage of your infant's diet is currently made up of breast milk?
_____ %

6. How old was your baby when you started feeding him/her formula?
___ ___ □ weeks
___ ___ □ months
6.1 What type of formula are you feeding your baby?
_____

7. If applicable, how old was your baby when you completely stopped feeding breast milk?
___ ___ □ weeks
___ ___ □ months
□ Not applicable

8. Has your baby had anything other than breast milk or formula to eat or drink?
□ Yes □ No
8.1 If Yes, what did the baby eat or drink? _____
_____

8.2 If Yes, how old was your baby when you first introduced other foods and/or beverages?

___ ___ □ weeks

___ ___ □ months

Appendix B

Participant Demographic Questionnaire

Subject ID: __ __ __ __
Date: __ __ / __ __/ __ __

Infant Health Information

1. Baby's date of birth: ___ ___ / ___ ___ / ___ ___ ___ ___

    1.1 Baby's current age: ___ ___wk ___/7 days

2. Baby's due date: ___ ___ / ___ ___ / ___ ___ ___ ___
3. Baby's sex: □ Female □ Male
4. Gestational age at birth: _____ wk _____/7 days
5. Birth weight: ___ ___ lb ___ ___ oz OR ___.___ ___ kg
6. Birth length: ___ ___.___ in OR ___ ___.___ cm

Maternal Health Information

7. How tall are you? ___ ft ___ ___ in OR ___ ___ ___ . ___ cm
8. What was your weight just before getting pregnant? ___ ___ ___ . ___ □ lb □ kg
9. Did you smoke during this pregnancy? □ Yes □ No →Skip to Question 10

    9.1 During which part of this pregnancy did you smoke? (check all that apply)

    □ First 3 months

    □ Middle 3 months

    □ Last 3 months

    9.2 About how many cigarettes did you smoke each day?

    □ < 1

    □ 1 - 9

    □ 10 - 19

    □ > 20

10. Are you currently smoking? □ Yes □ No →Skip to Question 11

10.1 About how many cigarettes do you smoke each day?

□ < 1

□ 1 - 9

□ 10 - 19

□ > 20

11. How many children do you have [specify age]?

Biological children:

_____

Adopted or foster children:

_____

Step children:

_____

12. Have you used any chemical hair treatments in the last 6 months such as hair color, chemical straightening, or permanent wave?

□ Yes □ No →Skip to Question 13

If yes, what type of chemical hair treatment(s) have you used?

_____

Race and Ethnicity

I would like to ask you some questions about your racial and ethnic background as well as the racial and ethnic background of your baby and the baby's father.

13. Are you, your baby, or your baby's father Hispanic or Latino? (check one per person)

	Mother	Baby	Baby's Father
Hispanic or Latino	□	□	□
Not Hispanic or Latino	□	□	□

14. If you had to choose ONE race to describe you, your baby, and your baby's father, which one would that be? (check one only per person)

	Mother	Baby	Baby's Father
American Indian or Alaskan Native	□	□	□
Asian	□	□	□
Black or African American	□	□	□
Native Hawaiian or Other Pacific Islander	□	□	□
White	□	□	□

Household Information

The next set of questions is about your family and household. Please think about your total household income during the last year. Include income from jobs, help from a family member or agency, disability, unemployment, child support, alimony, scholarships, social security, rents, or interest earned by any member of your household.

15. What was your total household income, before taxes?

□ < $19,999

□ $20,000 - $39,999

□ $40,000 - $59,999

□ $60,000 - $79,999

□ $80,000 - $99,999

□ > $100,000

□ Refused

□ Don't know

Mother's Information

Now I would like to ask some questions about you.

16. What is your date of birth? ___ ___ / ___ ___ / ___ ___ ___ ___

17. What is your marital status?

□ Married

□ Not married, but living with partner

□ Single

□ Divorced

□Widowed

18. What is your highest grade or year you completed school?

□ 8th grade or less (0-8)

□ Some high school (9-11)

□ High school graduate (12)

□ Some college/technical school (13-15)

□ Completed college (16)

□ Post graduate training/degree (17+)

19. What is your CURRENT employment status?

□ On maternity leave from employment: paid leave and benefits

□ On maternity leave from employment: benefits only

□ On maternity leave from employment: no benefits

□ Working full-time

□ Working part-time

□ Unemployed, no benefits

□ Unemployed, other benefits (e.g., Workers Compensation)

□ Other (specify): _____

Appendix C

Sensitivity Analysis of Weight-for-Length Percentile

Table 11. Multivariate predictors of infant hair Mn concentration (μg/g)[1], using weight-for-length percentile as predictor variable[2]

	β ± SE	*p*-value
	Model 1[3]	
Intercept	1.81 ± 0.87	0.05
Infant Hair As Concentration	0.46 ± 0.10	<0.001
Infant Hair Cd Concentration	0.03 ± 0.11	0.80
Infant Hair Pb Concentration	0.14 ± 0.13	0.30
Mother Hair Mn Concentration	0.25 ± 0.14	0.04
Mother Hair As Concentration	-0.001 ± 0.169	0.99
Mother Currently Smokes[4]	0.51 ± 0.38	0.19
Weight-for-Length Percentile	0.16 ± 0.09	0.09
	Model 2[5]	
Intercept	1.56 ± 1.04	0.15
Infant Hair As Concentration	0.48 ± 0.10	<0.001
Infant Hair Cd Concentration	-0.02 ± 0.12	0.88
Infant Hair Pb Concentration	0.13 ± 0.14	0.36
Mother Hair Mn Concentration	0.25 ± 0.11	0.04
Mother Hair As Concentration	-0.05 ± 0.18	0.78
Mother Smokes[3]	0.52 ± 0.37	0.18
Weight-for-Length Percentile	0.006 ± 0.003	0.07
Introduced to Complementary Feeding[6]	0.19 ± 0.18	0.30
Mother Smoked During Pregnancy[3]	0.33 ± 0.55	0.55
Infant Ethnicity[7]	-0.21 ± 0.60	0.73
Income[8]	-0.13 ± 0.07	0.05

[1]Hair metal levels log-transformed for this analysis; [2]See Table 10 for weight-for-length presented as z-score; [3]Includes variables with bivariate regression significance of $p < 0.05$; [4]Reference group is no smoking; [5]Includes variables with bivariate regression significance of $p < 0.15$; [6]Reference is complementary feeding not introduced; [7]Reference group is non-Hispanic; [8]Reference group is <$19,999.

Appendix D

Dietary Reference Intake for Manganese

Table 13. Adequate intake of manganese by life stage

Life Stage	Age	Men (mg/day)	Women (mg/day)
Infants	0-6 months	0.003	0.003
Infants	7-12 months	0.6	0.6
Children	1-3 years	1.2	1.2
Children	4-8 years	1.5	1.5
Children	9-13 years	1.9	1.6
Adolescents	14-18 years	2.2	1.6
Adults	≥19 years	2.3	1.8
Pregnancy	all ages	---	2.0
Lactation	all ages	---	2.6

Table 14. Tolerable upper intake level (UL) of manganese by age

Age	UL (mg/day)
0-12 months	*
1-3 years	2.0
4-8 years	3.0
9-13 years	6.0
14-18 years	9.0
≥19 years	11.0

*There is not enough evidence to set a UL for this age group. Breast milk and infant formula should be the only sources of Mn for this age group.