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BLOOD LEAD LEVELS, DIETARY INTAKE, AND OXIDATIVE STRESS IN LEAD-EXPOSED URUGUAYAN CHILDREN

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ABSTRACT

Lead exposure poses threats to health of millions of children worldwide. Weight of the evidence from experimental and epidemiological studies suggests that low-level lead exposure, a condition currently observed in general population, is associated with various adverse health outcomes in spite of the null findings in some studies. Although, we have a knowledge-base of the health consequences of exposure to lead in children, important questions particularly on the mechanisms of its toxicity, as well as prevention and treatment of lead exposure remain unanswered. Understanding the association between socio-demographic and dietary factors that could affect children's lead exposure and the relation between lead and oxidative stress, a proposed common mechanism for many lead-induced toxicities, could potentially help in finding effective preventive strategies for exposure and toxicities of low-level lead. This cross-sectional study in 211 children (5-8 years old) from Montevideo, Uruguay identified the potential socio-demographic and household risk factors that can predict children's blood lead levels (BLLs), a biomarker of lead exposure. The study also examined the relation between dietary intakes of nutrients and BLL, and investigated the association between BLL and two oxidative stress markers (measured as F_2- 8α isoprostane or isoprostane, a lipid oxidation marker and 8- hydroxy-2- deoxy Guanosine or 8-OH-dG, a DNA oxidation marker), and tested whether intakes of antioxidants act as effect modifiers. The mean BLL of the study children was 4.7 ± 2.2 µg/dL and 30.2% children had elevated BLL [≥ 5 µg/dL, the current reference level set by Centre for Disease Control for identification and monitoring of children who are exposed to more lead than most children; CDC, 2012]. The most salient socio-demographic and household risk factors for BLL in the study were: father's smoking, father's employment in jobs with potential for metal

exposure (such as construction, factories, and print shops, and as mechanics or drivers), and the number of young child (< 5 years old) in a household. Among dietary factors, carbohydrate (both as absolute amount and as percentage of energy intake) and fat (as % energy)—were positively associated with children's BLL. On the other hand, calcium intake was inversely associated with children's BLL. Majority of the study children did not meet the recommended intake for calcium (800 mg/day). Other nutrients were not associated with BLL and no interactions between pairs of nutrients (iron-zinc, iron-vitamin C or iron-calcium) on BLL were observed. Finally, a weak positive association was found between BLL and the urinary concentrations of isoprostane in the study children, but not between children's BLL and the concentration of urinary 8-OH-dG. The interactive effects of antioxidants (vitamin C or zinc) with BLL on oxidative stress markers were not statistically significant.

This study provides evidence that Uruguayan children continue to be exposed to lead years after the withdrawal of lead from gasoline. Because there is perhaps no safe level for lead and because low-level lead exposure may cause neurodevelopmental deficits and other long-term health problems, lead testing should be made a routine in pediatric practice in Uruguay. School entry is an opportune time for lead screening, if not done previously, since it can be conducted along with other screenings important for school achievement such as hearing and vision. Moreover, the identified socio-demographic and household factors in this study could be used for initiatives to make behavioral and lifestyle changes in children and families and to increase awareness among parents, health-care providers and policy-makers. Educational campaigns regarding prevention of lead exposure may potentially help children and their siblings. Finally, this study provides evidence that could be used to design future long-term studies to answer

questions regarding the biological mechanism and the role of nutrition in mitigating exposure and lead toxicities.

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Chapter 1

INTRODUCTION

Lead (Pb) exposure is one of the common environmental problems threatening the health of the children worldwide (Attina & Trasande, 2013; Bellinger, 2011; Lanphear, 2007; WHO, 2010). Incidents of fatal acute Pb poisoning have occurred in recent years (Dooyema et al., 2012), but they are rare. Current data from the US and around the world show that the majority of children suffer from chronic low-level environmental Pb exposure (Attina & Trasande, 2013; Jones et al., 2009; WHO, 2010). A review of pooled data of blood lead level (BLL), a biomarker of Pb exposure, from several studies conducted around the world between 2000-2010, shows that in most countries children's average lead levels in blood was less than 10 µg/dL (Attina & Trasande, 2013). In a nationally representative sample of US children (1-5 years), the geometric mean (95% confidence interval) blood lead concentration during 1999-2004 was 1.9 (1.8 – 2.1) µg/dL, with variations between geographic regions and comparatively higher BLL in minorities, children from low income families, and those living in older houses (Jones et al., 2009; Scott & Nguyen, 2011). The advisory committee on childhood lead poisoning prevention established by US Center for Disease Control (CDC) suggested a reference value of 5 µg/dL, based on the 97.5^{th} percentile of the distribution of children's BLL from the National Health and Nutrition Examination Survey (NHANES III), for identification of those with elevated BLL (CDC, 2012). Estimated from 2007-2010 NHANES data, about 535,000 US children less than 5 years of age had elevated BLL (Wheeler & Brown, 2013). Despite the low-levels of exposure, Pb toxicity is a

public health concern around the world due to a strong evidence of long-lasting adverse effects that Pb cause even at the lower levels. A summary on biology of Pb and the health effects of low-level lead exposure are reviewed below.

A brief overview on the biology of lead-

Exposure and absorption:

Lead (Pb), a heavy metal, is found in small amounts on earth's crust (ATSDR, 2007). It has many industrial usages due to its low melting point and excellent corrosion-resistant property (ATSDR, 2007). Due to the past and current global use (IARC, 2006) and its natural occurrence, Pb is present in the environment and people can be exposed to Pb through air, water, soil, dust, food and various other sources including paints and consumer products (ATSDR, 2007). Lead can be inhaled, ingested or absorbed through the skin. In children, ingestion of Pb is the primary route of exposure because of frequent hand-to-mouth or object-to-mouth behaviors (Mielke & Reagan, 1998).

Absorption of inhaled Pb through the pulmonary system depends mainly on the size of the Pb particles (Chamberlain, 1983). While most of the small Pb particles inhaled through the respiratory tract is readily absorbed into the blood, the large particles (greater than 2.5 μm diameter) deposit in the upper- respiratory tract, get transferred to the mouth by mucociliary action and are swallowed (ATSDR, 2007). Once ingested, Pb is absorbed through the gastrointestinal tract, but the mechanism of absorption is not fully understood. Two processes may be involved: (1) an energy-dependent, carrier-mediated active transport and (2) passive diffusion through the enterocyte. For the first mechanism, since Pb has no biological function in the body, the existence of a specific transporter for Pb is unlikely and one has not been

discovered so far. It is thought that Pb competes for the binding sites of the intestinal transporters that transport divalent nutrient-metals such as iron, calcium and zinc. The affinity towards sulfhydryl groups (-SH) and other organic ligands in proteins (Vallee & Umar, 1972) may be attributed to the ability of Pb to bind to the intestinal transporters. *In vitro* studies have shown that the divalent metal transporter 1 (DMT 1), the principal carrier of iron in the small intestine can transport Pb across the apical membrane (Bannon et al., 2002). However, it is important to note that DMT1 has a higher affinity for iron than Pb (Bannon et al., 2002) and a high level of DMT1 expression may be necessary for Pb transport (Bannon et al., 2003; Watson et al., 1986). The expression of DMT1 increases during periods of iron deficiency (Gunshin et al., 1998), allowing an increment in both iron and Pb absorption (Bressler et al., 2004; Kwong et al., 2004). Nonetheless, other studies have suggested DMT1-independent pathways for Pb absorption (Elsenhans et al., 2011; Fullmer 1991; Fullmer & Rosen, 1990) since the absence of DMT1 did not seem to alter intestinal Pb uptake (Bannon et al., 2003). This DMT1-independent pathway could be the vitamin D-dependent intestinal absorptive process of calcium which involves the entry of the lead into the enterocyte through the calcium channels, intracellular transport by binding to calbindin D and extrusion out of the absorptive cell and into the circulation via basolateral transporters (Fullmer et al., 1985; Fullmer & Rosen, 1990). In addition to the use of the absorptive pathways for iron and calcium, Pb-uptake may involve entry into the enterocyte via apical zinc-transporter ZIP4, followed by the transfer to the basolateral membrane by metallothionein (MT) and cysteine-rich intestinal protein (CRIP) (Jamieson et al., 2007) mostly due to lead's ability to bind with cysteine-rich zinc-binding sites (Godwin, 2001; Payne et al., 1999). Finally, an energy-independent intracellular or paracellular transport of Pb, driven by a concentration gradient from the luminal to the basolateral surface of the enterocyte, may be

possible (Mushak et al., 1991), but the hypothesis needs to be confirmed in *in vitro* and experimental studies. Questions remain as to whether these mechanisms work simultaneously or one mechanism is more prominent than others at a low versus high luminal concentration of Pb. Several factors such as dose (Sherlock & Quinn, 1986), particle size (Barltop & Meek, 1979; Grobler et al., 1988), solubility of Pb compounds (Barltop & Meek, 1979) as well as the physiological state (Rabinowitz et al., 1980; James et al., 1985), nutritional status (Blake & Mann, 1983; Heard & Chamberlin, 1982; Mahaffey, 1986), and age (Mushak et al., 1991) seem to affect the absorption of lead.

Distribution and excretion:

Once in the circulation, the majority of Pb (>99%) binds to proteins in the erythrocytes, the remaining small portion resides in the plasma bound to plasma proteins (ATSDR, 2007). The fraction of Pb in plasma is available to cross cell membranes and get entry into various organs in the body. While Pb in plasma may reflect "available" fraction of the current exposure, several analytical issues make it difficult to precisely measure the plasma Pb levels (Barbosa et al., 2005; Manton et al., 2001). Measurement of Pb in whole blood also referred as "blood lead level (BLL)" remains the most widely used and acceptable biomarker for assessing the exposure and Pb absorption in the body (Barbosa et al., 2005; IARC, 2006; WHO, 2010). Although between-and- within – individual differences in plasma to blood lead ratio exist, evidence suggests that these two biomarkers are highly correlated with each other (Smith et al., 2002). The clearance of Pb from blood varies from 28 to 36 days (Rabinowitz et al., 1976). While BLL is mostly a reflection of current exposure in children, it represents a steady state between recent external exposure and the internal deposits from the Pb released from bones in adults (Hu et al., 2007).

Lead can enter almost all tissues in the body including brain, kidney, liver—organs known as a target for Pb toxicity. Lead uses the voltage-regulated Ca^{2+} channel or the active transport system (such as Ca^{2+}-Mg^{2+}-ATPase) for crossing cellular membranes (Calderon et al., 1999). Due to the known neurotoxic effects, much attention has been paid to understanding how Pb enters and functions in the brain and neural tissues (Sanders et al., 2009; Lidsky & Schneider, 2003). Lead may get entry into the brain by crossing the blood-brain barrier (BBB), an interface between blood, brain and cerebrospinal fluid, through calcium channels and/or increasing the permeability of the BBB by decreasing the expression of the tight junction proteins (Struzynska et al. 1997; Yokel, 2006). It can get transported to astrocytes by DMT1 or by other unidentified mechanisms (Cheong et al., 2004). Alternatively, the entry of Pb into the astrocytes and neurons may occur via voltage-sensitive calcium channels (Kerper & Hinkle, 1997). Because the BBB is not as developed in children as in adults and because childhood is a period of critical and rapid development of the nervous system, children are vulnerable to Pb accumulation in the brain and its neurotoxicity (Sanders et al., 2009).

Bone, on the other hand, acts as a major repository for Pb by substituting calcium in the hydroxyapatite particularly during the periods of bone deposition (Rabinowitz et al., 1976). The half-life of Pb in bone can vary between years to decades (~25 years). Majority of Pb in bone accumulates in the storage compartments i.e. cortical and trabecular bones, while a small amount resides in the compartments that rapidly exchange minerals with extracellular fluids and plasma (Hu et al., 2007). Bone formation and resorption are critical for the exchange of Pb between blood and bone tissues. Due to the higher rate of bone resorption, bone-to-blood mobilization of Pb increases during the periods of pregnancy (Gulson et al., 2003), lactation (Ettinger et al., 2007), menopause (Garrido-Latorre et al., 2003) and advanced age (Hu et al., 1996). Because Pb

can cross the placental membranes by passive diffusion during pregnancy (Goyer, 1990) and can get excreted in breast milk (Koyashiki et al., 2010), the mobilization of maternal bone Pb deposits into the circulation is a significant endogenous source of pre- and early-postnatal exposure for the fetus and nursing infants, respectively (Gulson et al., 2003; Ettinger et al., 2007).

Lead is excreted primarily through the urine and feces with small percentage of elimination also occurring via sweat. Urinary excretion represents mostly the plasma Pb that is filtered and excreted through the kidneys (Barbosa et al., 2005). Because of non-invasive nature and ease of collection, measurement of Pb concentration in urine (U-Pb) has been considered, but several biological and analytical issues preclude U-Pb from being a preferred biomarker. For example, the concentration of Pb in urine can be influenced by variation in individual hydrations status that necessitates a creatinine excretion correction or density adjustment. In addition, urinary Pb level is closely correlated with plasma Pb than with the whole blood Pb because majority of Pb in blood binds to the proteins in the erythrocytes with a small portion residing in plasma and a fraction of Pb that has diffused from plasma is excreted through the kidneys. Thus, urinary Pb may underestimate the magnitude of exposure. Finally, several methodological problems exist for the analysis of U-Pb including reliable quality control and certified reference materials, and precipitation of urate salts during storage (Barbosa et al., 2005).

Adverse health effects of low-level Pb exposure-

Several studies in the last decade illustrated toxic effects of Pb in both children and adults at lower levels of exposure (for review see Bellinger, 2011; Navas-Acien et al., 2007; Rossi, 2008), while some did not (IARC, 2006; Lustberg & Silbergeld, 2002; Wolf et al 1994). Despite

the continuing debate on the potential effects of low-level Pb exposure (Gidlow, 2004), the weight of the evidence tends to suggest that even at a low-level Pb is associated with children's neurobehavioral development and other health outcomes in both children and adults. Unlike in acute fatal Pb poisoning, where overt clinical symptoms are presented, no specific symptoms exist at a lower range of exposure, making the condition difficult to diagnose. Thus, low-level Pb exposure is a public health concern that poses difficulties to identify, monitor and prevent. However, analysis of existing evidence from studies showing adverse effects at low levels suggests that if not managed the toxic effects of Pb could cause substantial economic and societal burden (Attina & Trasande, 2013; Gould, 2009).

In children:

The toxic effects of high level Pb exposure have been known for centuries (Lewis, 1985; Needleman, 1999). It is the new evidence that has emerged in the last decade on the deleterious effects of Pb in both children and adults at a low range of exposure previously thought to be safe is a cause of concern for researchers, policy makers and health-care providers (Bellinger, 2011). Still, disagreement persists on how low is "low-enough" with regard to neurocognitive effects of Pb observed in young children (Bellinger, 2008). As the BLLs in general population started to decline both in the US and around the world, mainly due to withdrawal of Pb from gasoline, studying effects of Pb at lower levels became feasible. Many studies found decreased cognitive functions and behavioral problems in children with BLL below 10 µg/dL (Canfield et al., 2003; Chiodo et al., 2004; Surkan et al., 2007), while in some studies children had a mean BLL as low as 1-2 µg/dL (Emory et al., 2003; Jedrychowski et al., 2008). Not only do studies support the existence of a positive association between BLL and children's neurobehavioral development,

the effects may be greater at a lower range of BLL (Bellinger et al., 1992; Canfield et al., 2003; Chiodo et al., 2004; Kordas et al., 2006; Lanphear et al., 2000; Schwartz, 1994). For example, in a prospective study with 0-5 years old children, an increase in 10 µg/dL in the life time average BLL was associated with a 4.6 points decrease in children's intelligence quotient (IQ) scores, whereas a loss of 7.4 IQ points was found for an increase in BLL from 0 to 10 µg/dL (Canfield et al., 2003). An analysis of pooled data from seven international prospective cohort studies found a decline in children's intelligence quotient (IQ) scores by 3.9, 1.9 and 1.1 points for an increase in BLL from 2.4 to 10 µg/dL, 10 to 20 µg/dL and 20 to 30 µg/dL, respectively, suggesting a steepest decline in IQ at BLL< 10 µg/dL (Lanphear et al., 2007). Due to a lack of plausible explanations on the biological mechanism behind the non-linear shape of this dose-response relationship, it is hypothesized that within the range of exposure that does not result in overt poisoning, an increase in BLL beyond a certain level may cause very little additional impairment in children's cognitive function (Bellinger, 2008).

In addition to IQ, Pb-associated deficits have been reported in other domains of cognitive function such as visuo- spatial skills, executive functions, fine and gross motor skills, memory, problem solving and academic skills like reading, math and spelling in children with BLL lower than 10 µg/dL by some studies (Bellinger et al., 2008; Kordas et al., 2006; Miranda et al., 2007). Apart from cognitive functions, blood Pb concentration has also been associated with a wide range of behavioral problems including inattention (Bellinger et al., 1994; Chiodo et al., 2007; Roy et al., 2009), disorganization (Bellinger, 2004), distractibility, hyperactivity (Canfield et al., 2004; David et al., 1972; Lansdown et al., 1986; Needleman et al., 1979) and off-task behaviors (Chiodo et al., 2004; Kordas et al., 2007) in some studies. While evidence is beginning to emerge showing Pb exposure as a risk factor for Attention Deficit Hyperactivity Deficits (ADHD)

(Braun et al., 2006; Froelich et al., 2009; Nigg et al., 2008; 2010) even in children with BLL lower than 5 µg/dL, current data is insufficient to support the hypothesis (Eubig et al., 2010).

The persistent nature of Pb neurotoxicity is also evident as early-life Pb exposure is linked with cognitive deficits and behavioral abnormalities into adult years by some researchers (Mazumdar et al., 2011; Nevin, 2007; Wright et al., 2008). One study examined the intellectual functions of adults (average age 29 years) from a sub-sample of participants in the Boston prospective study who were enrolled into as infants and followed up to the age of 10 years (Mazumdar et al., 2011). After adjusting for potential confounders, a strong relationship was found between the adult IQ test scores and BLL at six months (mean BLL: 8.0 ± 5.3 µg/dL), four years (mean BLL: 6.7 ± 3.6 µg/dL), 10 years (mean BLL: 3.0 ± 2.7 µg/dL) and with average late childhood BLL (mean of BLL at four and 10 years) (Mazumder et al., 2011). In another prospective cohort study, higher prenatal (mean BLL: 8.3 ± 3.8 µg/dL), average childhood BLL (defined as mean of BLL from 3 months through 6 years of age: 13.4 ± 6.4 µg/dL) and the concurrent BLL at 6 years (mean BLL: 8.3 ± 4.8 µg/dL) were all significantly associated with higher rates of criminal arrests in adults (average age: 22.5 years) (Wright et al., 2008). Other behavioral problems including hyperactivity, inattentiveness, failure to graduate from high school, conduct disorder, delinquency and drug use in adults have been associated with childhood low-level Pb exposure as indicated by several studies (Braun et al., 2006; Dietrich et al., 2001; Fergusson et al., 2008; Ha et al., 2009; Needleman et al., 1990, 1996, 2002; Nigg & Casey, 2005; Nigg et al., 2008; Sciarillo et al., 1992;; Wang et al., 2008; Wright, 2008). Based on these results, some researchers suggested that LOAEL (lowest observable adverse effect level) for Pb are perhaps equal to zero particularly for developmental neurotoxicity (Rogan & Ware, 2003; Ronchetti et al., 2006).

Apart from neurobehavioral deficits, a few studies also reported decreased growth and hearing acuity, delayed sexual maturation, and dental caries in relation to low-level lead exposure in children (Bellinger, 2011). A significant association was observed between BLL and reduced weight, height, and the lengths of trunks, legs and arms in school-age children with a mean BLL< 10 µg/dL in one study (Ignasiak et al., 2006). Lead level in children was also associated with hearing loss in children with a mean BLL less than 10 µg/dL (Schwartz & Otto, 1991). A positive association between BLL and the development of dental caries was found in groups of children with a mean BLL< 5 µg/dL (Gemmel et al., 2002; Moss et al., 1999). Moreover, a significant association of BLL has been observed with delayed development of pubic hair and onset of menarche in girls (Wu et al., 2003) and a delay in the onset of puberty in boys with a mean BLL< 10 µg/dL (Hauser et al., 2008). However, more evidence is needed to infer causality and support the hypothesis that Pb contributes to these health problems even at a low-level.

In adults:

Low-Pb exposure is not only a problem in children, some studies show detrimental health outcomes in both occupationally and non-occupationally exposed adults (Bellinger, 2011). Although mixed results exist and the evidence is not sufficient for many health outcomes, emerging studies have started showing relations between chronic low-level Pb exposure and health outcomes beyond developmental neurotoxicity. Bellinger (2011) summarized the epidemiological evidence on the health effects of Pb in adults, focusing on the lowest observed levels of adverse effects. Some studies suggest an association of low-level Pb with overall mortality (Khalil et al 2009; Weisskopf et al 2009; Schober et al 2006; Menke et al 2006),

mortality and morbidity due to cardiovascular diseases (Menke et al., 2006; Navas-Acien et al., 2007), cancer (Alatise & Schrauzer, 2010), renal dysfunction (Muntner et al., 2003; Navas-Acien et al 2009; Tsaih et al., 2004), reproductive (Rothenberg et al., 2002; Sowers et al., 2002) and cognitive problems (Bakulski et al., 2012; Schwartz et al., 2005; Shih et al., 2007) in both occupationally and environmentally exposed adults. In terms of all-cause mortality and deaths due to cardiovascular disease, the lowest observed levels of adverse effects seem to be below the BLL of 10 µg/dL in adults (Menke et al., 2006; Schober et al., 2006). On the other hand, despite a modest strength of association, several epidemiological and experimental studies show compelling evidence for a link between long-term Pb exposure and elevated arterial pressure (Navas-Acien et al., 2007). However, the lowest level beyond which Pb not associated with elevated blood pressure is unknown, current evidence may suggest it is as low as 3-5 µg/dL (Martin et al., 2006; Vupputuri et al., 2003). Similarly, many studies have shown higher likelihood of chronic kidney disease with higher BLL in both general adults and particularly in patients with kidney dysfunctions, hypertensions and diabetes (Bellinger, 2011; Ekong et al., 2006). The association between Pb and reduced glomerular filtration rate (GFR) has been observed in cohort of adults with as low mean BLL as 2 µg/dL (Akesson et al., 2005; Muntner et al., 2003). In addition to neurodevelopmental effects in children, bone Pb (but not always BLL) have been associated with adverse effects in the central and peripheral nervous systems (CNS and PNS) in adults with mean BLL as low as about 3 µg/dL (Iwata et al., 2005; Khalil et al., 2009; Sanders et al., 2009; Shih et al., 2007; Weuve et al., 2009). While poorer performance in cognitive tests examining learning, memory and attention have been associated with bone Pb and/or BLL in elderly people with current mean BLL below 10 µg/dL (Weisskopf et al., 2004; Wright et al., 2003), some studies did not observe such associations at similar levels of exposure

(Krieg et al. 2005; Nordberg et al., 2000). Moreover, increased risk for development of neurodegenerative diseases such as Parkinson's and Alzheimer's have been linked with chronic Pb exposure measured as Pb levels in bone (Bakulski et al., 2012; Monnet et al., 2006; Weisskopf et al., 2010). Since the correlation between bone Pb and BLL is not high, it is difficult to compare the results across the studies and estimate the lowest level for Pb-associated cognitive deficits in adults (Bellinger, 2011). There is some evidence of reduced fertility and pregnancy complications like eclampsia in women with BLL< 20 µg/dL (Alexander et al., 1996; Sallmen et al., 2000; Shiau et al., 2004) and limited evidence for the risk of increased spontaneous abortion with BLL< 30 µg/dL (CDC, 2010). Maternal bone Pb at a level relevant to the range observed in general population and BLL below 10 µg/dL have been linked with fetal growth in some studies (Hernandez-Avila et al., 2002; Sanin et al., 2001; Zhu et al., 2010). Because of the potential reproductive effects, CDC recommends monitoring and interventions of pregnant women with BLL> 5 µg/dL (CDC, 2010). In contrast, the evidence regarding carcinogenicity of Pb in humans is limited with inconsistencies observed in findings across the studies (IARC, 2006). While previous studies in humans did not report significant associations between Pb exposure and incidents of cancer, some recent reports show higher BLL and hair Pb in cancer patients compared to their controls (Alatise & Schrauzer, 2010; Bhatti et al., 2009). One critical consideration that needs to be given while examining the health consequences of Pb exposure in adults is the effects of both current and past (cumulative) exposure, because Pb gets deposited in bones and can remain there for decades (Hu et al., 2007).

Based on the existing evidence particularly on neurotoxic effects in children and cardiovascular effects of low-level Pb exposure in adults, the FAO/WHO joint committee on food additives and European food safety authority's panel on the contaminants in the food chain

concluded that there is no indication of a safe threshold for adverse effects of Pb and withdrew the Provisional Tolerable Weekly Intake (PTWI) of 25 μg Pb/kg body weight (EFSA, 2011; FAO, 2010).

Mechanism of toxicity:

The underpinning of Pb toxicity is not clearly understood. Multiple cellular and molecular mechanisms that can affect many enzyme systems and cellular processes are involved in the various toxic effects of Pb at various sites in the body. The major toxic effects of Pb are observed on the hematological, neurological, cardiovascular and renal systems. Although one mechanism cannot explain all the observed toxic effects of Pb, oxidative stress seems one common mechanism by which Pb is thought to induce its toxic actions on various organs (Ahamed et al., 2007; Bellinger, 2008; Sanders et al., 2009; Vaziri et al., 2008). At the molecular level, the primary mode of action for Pb is attributed to the metal's high affinity for thiol groups (-SH) and other organic ligands in proteins (Vallee & Ulmer, 1972). Another characteristic of Pb is the ability to substitute for other biologically essential metals such as calcium, iron, and zinc in the cell molecular machinery (Sanders et al., 2009). Using these properties, Pb may induce oxidative stress at the cellular level by generating free radicals including reactive oxygen species (ROS), depleting endogenous antioxidant gluthathione (GSH) and changing the activities of the antioxidant enzymes (Ahamed et al., 2007; Sanders et al., 2009). Lead can produce free radicals indirectly by inhibiting the activity of the hemopoeitic enzyme Amino Levulinic Acid Dehydratase (ALAD). By substituting zinc and binding to the sulfhydryls at the active site of ALAD, Pb inhibits the function of the enzyme (Warren et al., 1998). The ALAD catalyzes the conversion of aminolevulinic acid (ALA) to porphobiliniogen, a precursor of heme (Kappas et

al., 1995). The inhibition of ALAD by Pb results in the accumulation of the substrate, δ-aminolevulinic acid (δ-ALA). This is a very unstable compound and can be rapidly converted into free radicals (Gurer-Orhan et al., 2004). Moreover, the accumulation of Pb in the mitochondria and subsequent damage to the membrane can increase the entry of calcium (Ca^{2+}) into the mitochondria. An increased entry of Ca^{2+} can enhance the mitochondrial electron transport, thereby increasing the production of reactive oxygen species (Sanders et al., 2009; Lidsky & Schneider, 2003). The affinity of Pb towards sulfhydryl groups (-SH) makes GSH more susceptible to binding with Pb, which can result in inactivation of the antioxidant action of GSH (Hoet, 2005; Patra et al., 2000). Moreover, Pb can alter the activities of antioxidant enzymes by several mechanisms including replacing and forming complexes with the cofactors required for the functioning of these enzymes. For example, by forming a complex with selenium, Pb can decrease the enzymatic activity of GPx (Whanger, 1992). The replacement of Cu and Zn by Pb may decrease the activity of the Cu/Zn-dependent SOD (Mylorie et al., 1986). In addition to generating free radicals and altering the antioxidant system, Pb can produce direct peroxidative damage to the cell membrane by producing changes to the protein and fatty acid composition of the lipids in a way that makes the membrane more vulnerable to oxidation (Lawton & Donaldson, 1991). While all of these mechanisms are possibly involved in the development or exacerbation of oxidative stress from exposure to Pb, it is not clear whether and which mechanism operates at different levels of exposure.

As a secondary response to Pb-induced oxidative stress, the availability of biologically active nitric oxide (NO) and the production of cyclic guanosine monophosohate (cGMP) are reduced (Vaziri et al., 2008). The vasodilatory actions of NO play a key role in the regulation of blood pressure. Functional deficiency of NO along with the production of highly cytotoxic

peroxynitrite can contribute to the adverse cardiovascular, renal and neurological consequences of Pb exposure (ATSDR, 2007; Vaziri et al., 2008). In addition, the decreased production of cGMP increases cytosolic concentration of calcium ions (Ca^{2+}) in vascular smooth muscle cells. This, in turn, increases vascular resistance and subsequently the arterial pressure (Vaziri et al., 2008). Oxidative stress can also trigger a cascade of cellular events including the development of inflammation (which promotes cardiovascular diseases) (Vaziri et al., 2008), activation of apoptosis and excitotoxicity leading to neuronal deaths (Sanders et al., 2009). Apart from oxidative stress, the mechanism of lead-induced neurotoxicity could be attributed, in part, to the disruption of calcium-mediated processes in the central nervous system (Bressler & Goldstein, 1991; Garza et al., 2006; Sanders et al., 2009). For example, Pb activates and modifies the calcium-binding protein calmodulin (Kern et al., 2000) and protein-kinase C (PKC) (Hwang et al., 2001; Garza et al., 2006). Alterations in the structures and functions of these proteins can compromise the second messenger systems within the cells, leading to changes in gene expression, protein synthesis and release of neurotransmitters (Bressler & Goldstein, 1991; Garza et al., 2006; Sanders et al., 2009). In addition, other actions of Pb in the nervous system includes apoptosis or programmed cell death, mitochondrial dysfunction, disruption in storage and release of neurotransmitters, alterations of neurotransmitter receptors, second messengers, cerebrovascular endothelial cells and glial cells (Garza et al., 2006; Lidsky & Schneider, 2003; Sanders et al., 2009).

Genetic susceptibility to bioaccumulation and Pb toxicity:

Evidence shows that polymorphisms in at least three genes (ALAD, vitamin D receptor or VDR and hemochromatosis or HFE) can potentially affect bioaccumulation and toxicity of Pb

(Kelada et al., 2001; Onalaja & Claudio, 2000). As discussed before, ALAD is a hemopoeitic enzyme that is subject to inhibition by Pb. A polymorphism of ALAD gene produces two alleles- ALAD-1 and ALAD-2, which are inherited in a codominant pattern (Battistuzi et al., 1981). Some studies show that individuals with ALAD-2 phenotype have higher BLL than those with ALAD-1 possibly due to the higher affinity of ALAD-2 protein for Pb (Kelada et al., 2001; Scinicariello et al., 2007). On the other hand, adolescents with ALAD-2 homozygotes performed better in cognitive tests than ALAD-1 homozygotes (Bellinger et al., 1994). Recent studies provide further evidence for modification of the relation between blood and bone Pb levels, and cognitive functions in children and older adults with low-Pb exposure (Pawlas et al., 2012; Rajan et al., 2008)

The vitamin D receptor gene encodes the vitamin D receptor protein that binds to the active form of vitamin D (calcltriol) in the nucleus of the intestinal cells and other tissues such as bone and kidney. This, in turn, activates the genes encoding the calcium-binding proteins such as calbindin-D, which is responsible for calcium transport across the intestinal cells, and other calcium-rich tissues such as bones (Onalaja & Claudio, 2000). At least two alleles (b and B) and three genotypes (bb, Bb and BB) of VDR have been identified. Lead uses the vitamin D-dependent intestinal absorptive process of calcium for absorption through gastrointestinal tract. Therefore, a genetic polymorphism that modifies calcium absorption may modify lead absorption and distribution. Highest levels of blood and tibia Pb have been observed in occupationally-exposed adults with the BB homozygous than those with Bb and bb genotypes (Schwartz et al., 2000). Blood Pb concentration of environmentally-exposed children has also been shown to be affected by VDR gene polymorphisms (Chen et al., 2010; Haynes et al., 2003). Some studies suggest that genetic variation may influence maternal blood and umbilical cord Pb levels

(Ettinger et al., 2006; Rezende et al., 2010). Furthermore, performances on various cognitive tests as a function of BLL varied by VDR genotypes in children and adults, suggesting some genetic modifications of Pb- neurotoxicity (Krieg et al., 2010; Pawlas et al., 2012). More research is needed to understand the effects of VDR polymorphisms on absorption and distribution of Pb and whether the toxicity differs due to individual variation in VDR genotype.

The hemochromatosis (HFE) protein is a trans-membrane protein, found mostly in liver and intestinal cells and regulates the iron absorption by binding to the transferrin receptor and inhibiting the receptor's affinity to transferrin-loaded iron. HFE also regulates the production of hepcidin (produced in the liver), which controls iron absorption and iron homeostasis in the body (Pantopoulos, 2008). Two common polymorphisms of this gene, HFE C282Y and H63D are associated with hemochromatosis, an autosomal recessive genetic disease that causes an increase in absorption of ingested iron and iron overload in the body leading to tissue damage (Hanson et al., 2001). Since iron and Pb competes for intestinal absorption and uptake into other tissues such as brain, variations in iron metabolism gene like HFE could modify Pb absorption and neurotoxicity. Limited evidence suggests that variations in HFE gene may also affect body Pb burden and increase susceptibility to adverse effects such as cognitive and cardiac functions (Hopkins et al., 2008; Onalaja & Claudio, 2000; Wang et al., 2008; Weuve et al., 2006; Wright et al., 2004; Zhang et al., 2010).

Although research has provided some understanding on how nutritional and genetic factors independently influence Pb absorption, distribution and toxicity, gaps in knowledge still exist. Moreover, nutrient-gene interactions and their effects on Pb absorption, accumulation and toxicity need to be examined in animal models and epidemiological studies.

Current reference level:

Cumulative evidence on the health effects at low-level Pb exposure has prompted the US Center for Disease Control (CDC) to lower the reference level to identify children with elevated BLL from 10 to 5 µg/dL in 2012 (CDC 2012). In addition, the term "level of concern" has been replaced by "reference level" for monitoring, risk assessment and management purposes (CDC 2012). The CDC has based the reference value on the 97.5^{th} percentile of the blood Pb distribution of children participating in NHANES (2007-2010). Children with BLL≥ 5 µg/dL are considered exposed to more Pb than most children in a population. However, this reference level has no special biological relevance with regard to Pb toxicity. The natural "background" level of blood lead in preindustrial humans is estimated to be close to 0.016 µg/dL (Flegal & Smith, 1992), two fold lower than 1-2 µg/dL, the lowest mean BLL at which significant association has been observed between Pb and neurobehavioral problems (Emory et al., 2003; Jedrychowski et al., 2008).

Rationale for the study undertaken in this dissertation-

Over the years, an extensive body of literature has contributed to our understanding of the exposures, sources and health effects of lead. Yet, important gaps in knowledge still exist and these include: (a) what is/are the biological mechanism/s behind lead-induced toxicities; (b) whether children's nutritional status can modify exposure and toxic effects of lead; (c) what constitutes optimal and cost-effective strategies for the prevention and treatment of lead exposure and toxicities, especially at the low spectrum of environmental exposure; and (d) what is the role of nutrition in general and any components in particular in reducing the exposure and effects of lead. Because abating lead from the environment, a primary control process, is economically

challenging, there is a need for continued research to find cost-effective prevention and intervention strategies against lead exposure. Answering the above questions can help in formulating effective secondary preventive strategies against childhood lead exposure.

Children are at risk for exposure to lead from multiple sources, including lead in the air (from industrial use), vehicular emissions (combustion of leaded gasoline), water supplied through leaded pipes, leaded paint, lead-glazed ceramics, lead in the soil and dust from gasoline, past or present industrial activities (Mielke, 2002; Mielke & Regan, 1998). Some socio-demographic factors predictive of children's BLL are socio-economic status, parental smoking, and educational status of the parents (Liu et al., 2012; Nriagu et al., 2011; Queirolo et al., 2010; Disalvo et al., 2009). Although there are some common predictors of BLL, country and region specific differences exist. Identifying potential factors contributing to BLL in a population is important for future targeted intervention efforts, including communication of the exposure risk and awareness among parents and communities.

Apart from socio-demographic, environmental and household factors, children's nutritional status has been proposed as an important moderator of lead exposure. Deficiencies or excess of nutrients can predispose children to lead exposure and its toxic effects. For example, studies have shown that iron-deficient children are at greater risk for having elevated BLL than children who have adequate iron nutrition (Bradman et al 2001; Kwong et al 2004; Wright et al 2003). Higher calcium and zinc intake have also been associated with lower BLL in some studies (Elias et al., 2007; Lacasaña et al., 2000; Schell et al., 2004). The relation between macronutrients (carbohydrate, protein, fat) and BLL is mostly unknown, with some studies finding higher fat intake to be associated with higher BLL (Gallichio et al., 2002; Lucas et al.,

1996). In contrast, the findings regarding protein intake are conflicting, with some studies reporting a positive association of protein intake and BLL in infants (Penuela et al., 2006; Schell et al., 2004) and others observing negative or no association (Lucas et al., 1996; Quarterman et al., 1978; Mooty et al., 1975). The consumption of tofu, a good source of protein, has been inversely associated with BLL in Chinese adults (Chen et al., 2001). Nevertheless, literature regarding macronutrient intake and BLL is insufficient and further investigation is warranted. So far, supplementation with nutrients such as iron alone (Wolf et al., 2003; Zimmerman et al., 2006), iron and zinc (Rosado et al., 2006) or calcium (Canfield et al., 2005; Keating et al., 2011) has had limited success in lowering children's BLL. The relationship between BLL and the dietary intake of nutrients is not well characterized, but would be required for establishing an optimum dietary strategy.

For formulating effective preventive/therapeutic strategy, it is also important to understand the biological underpinnings of lead toxicity. As discussed before, oxidative stress, an imbalance between pro-and anti-oxidants with increase in the former, has been proposed as one molecular mechanism for lead-induced toxicities (Ahamed & Siddiqui, 2007). Evidence supporting the hypothesis that lead exerts oxidative stress mostly comes from the experiments in animals (Berrahal et al., 2009; Haleagrahara et al., 2011; Sandhir et al., 1994) and occupationally-exposed adults with high levels of lead exposure (Grover et al., 2010; Permpongpaiboon et al., 2011; Prokopowicz et al., 2013). The question remains whether lead exposure, at a level generally observed in a population, is associated with oxidative stress. In addition, few studies have examined this relationship in children (Ahamed et al., 2008; Cabral et al., 2012; Jin et al., 2006; Mielzynska et al., 2006). The role of nutrients, particularly that of

antioxidants, as a moderator of lead-induced oxidative stress has not been well studied, but can be critical in preventing toxicity (Gurer & Ercal, 2000; Psu & Guo, 2002).

Identification of the factors predictive of children's lead exposure such as socio-demographic and household behaviors, assessment of the nutrients as potential moderator for childhood lead exposure and greater knowledge of the molecular mechanisms behind lead toxicities- all of these may ultimately contribute to a better risk assessment and prevention against lead exposure. Although most of the countries have banned leaded gasoline (UNEP, 2012), the use of lead for industrial purposes has increased over the years (International Lead and Zinc Study Group, 2009; WHO, 2010). As a result, millions of children worldwide continue to be chronically exposed to low-level environmental lead and the problem is far from resolved (Bellinger, 2011). Therefore, strategies that modulate the exposure, body burden and the effects of lead in children are of public health significance.

To better understand the factors that have the potential for providing safeguards to children against lead exposure, a cross-sectional study was conducted in Montevideo, Uruguay. This study was set to examine the relation between dietary intakes of nutrients and BLL, to investigate the association between BLL and oxidative stress, and to test whether intakes of antioxidants can act as effect modifiers in the association between BLL and oxidative stress in 5-8 years old school children exposed to low-levels of lead. Additionally, we aimed to identify the potential socio-demographic and household risk factors that predict children's BLL. The primary rationale behind the study was to provide preliminary information on the relationship between nutritional status and lead exposure, and the pro-oxidative effects of low-level lead exposure in school children. Given the high cost and logistics of clinical trials and longitudinal studies, the

information from this study will be crucial in providing directions to future mechanistic studies and dietary interventions in children to identify a secondary method to prevent childhood lead exposure. Because this study was conducted in school-age children and because school entry is a crucial time for social, behavioral and cognitive growth, findings of this study have important implications for children's development as well.

Suitability of the study setting:

The study was conducted in Montevideo, the capital of Uruguay. Montevideo has a population of 1.3 million (Censos 2011). In 2011, the number of school-age (5-19 years) children in Montevideo was 265,405 of which about 83, 870 belonged to the age group of 5 to 9 years (Censos 2011). The presence of lead exposure in Uruguayan children has been documented in the past (Cousillas et al., 2005; Schutz et al., 1997). In 2001, the detection of high BLL (> 20 µg/dL) in impoverished children from La Teja neighborhood of Montevideo caused a huge media and public outcry (Mañay et al., 2008). This incident led to a popular belief that lead exposure is only limited to certain neighborhoods of Montevideo and only among low-socioeconomic populations. To the contrary of this perception, Queirolo and colleagues (2010) reported a high prevalence of elevated BLLs (5 µg/dL; the current reference level set by Center for Disease control for identifying, monitoring and managing children with elevated BLL; CDC 2012) among preschool children from several neighborhoods of Montevideo. Although the country has banned lead from gasoline in 2004, exposure from other sources continues (Mañay et al., 2010). Potential sources of lead exposure in Montevideo include lead processing industries such as foundries, manufacturing and recycling of batteries, water supplied through old leaded pipes, and the use of lead-based paints (Mañay et al., 2008). Furthermore, many lead-emitting

industries in Montevideo are located within residential areas, posing a significant risk of exposure to the surrounding populations (Mañay et al., 2008). In Montevideo, some detrimental effects of lead exposure on cognitive and behavioral development of young children have been reported (Kordas et al., 2012, 2011). One study examined the association between hemoglobin concentration, BLL and neuro-cognitive development in preschool children (Kordas et al., 2012). Kordas and colleagues (2012) observed that increasing hemoglobin concentration was associated with better performance in infant developmental scale in children with lower BLL (<5 μg/dL) but not with elevated BLL (≥ 5 μg/dL), suggesting that the developmental benefits of having better hemoglobin status may be attenuated by children's lead exposure. Moreover, lead exposure in Uruguayan mothers or young children may affect maternal parenting style, with elevated BLL (≥ 5 μg/dL) being associated with perceived difficulties in setting limits and discipline by the mothers, which may influence children's neurobehavioral development (Kordas et al., 2011). As a part of the cross-sectional study conducted in Montevideo school children, psychological assessments of the children were undertaken by Kordas and colleagues to examine the relation between lead exposure, cognition, behavior and academic achievement. In future, the findings of this investigation will shed light on the functional effects of lead exposure in these children.

According to a cost-estimate analysis, the majority of the economic burden due to loss of IQ associated with childhood lead exposure is borne by populations of low-and middle-income countries like Uruguay (Attina & Trasande, 2013). These authors calculated a 2.6% loss in GDP (gross domestic product- an indicator of a country's economic growth) and a loss of economic productivity equal to $1.4 billion for Uruguay due to lead-associated cognitive deficits in children (Attina & Trasande, 2013). Montevideo is a unique setting for the current study because

the children here have been exposed to low-levels of environmental lead, commonly observed in general population around the world. In addition, there is a lack of studies in this population, and blood lead testing is not a routine occurrence in pediatric practice. We have a limited understanding of the exposure characteristics and effects of lead exposure in these children. Being a developing country, Uruguay also faces the economic and logistic challenges of controlling and preventing lead exposure. There is a lack of public awareness, policies and programs on environmental exposure in Uruguay. The findings of this study can provide evidence, inform policies and help formulate strategies to prevent lead exposure in Uruguayan children.

Objectives and hypotheses-

The specific objectives of the current study were:

1. To examine the socio-demographic and household behavioral factors related to BLL in first-grade children living in several neighborhoods of Montevideo, Uruguay.

   Based on previous work in infants in this population, we *hypothesize* that low-socioeconomic status (SES), parent's involvement in jobs with Pb exposure, and mother's smoking status will positively predict children's BLL.

2. To determine the association between nutrient intakes and BLL in first grade children focusing on macronutrients (carbohydrate, protein, fat) and select micronutrients (calcium, iron, zinc, vitamin C and folate).

   The working *hypothesis* is that higher intakes of macronutrients and lower intakes of micronutrients will be associated with higher BLLs.

3. a. To investigate the relation between BLL and oxidative stress markers (measured as F_2-8α isoprostane, a lipid oxidation marker and 8- hydroxy- 2- deoxy Guanosine, a DNA oxidation marker) in first-grade children.

   We *hypothesize* that higher BLL will be associated with higher urinary concentrations of F_2- 8α isoprostane (isoprostane) and 8- hydroxy- 2- deoxy Guanosine (8-OHdG).

   b. To examine potential effect modification of the lead-oxidative stress association by vitamin C and zinc intake.

   The working *hypothesis* is that the predicted increase in the concentrations of urinary isorprostane and 8-OHdG with an increase in BLL will be lower in children with higher intakes of vitamin C and zinc compared to those with lower intakes of the two nutrients.

These three specific aims were addressed in detail in chapters 3, 4 and 5. Each chapter will provide the background, methodology, results and a detailed discussion on the findings of the studies that addressed the objectives above. Fulfilling the objectives will add knowledge to the existing literature on lead, particularly about potential secondary preventive approaches that could benefit lead-exposed children worldwide. This study will also inform the design of prospective studies in children to achieve the long-term goal of understanding the mechanism behind lead toxicity, and whether nutrition can play any role in reducing lead exposure and toxicity. Findings from this study can be useful in informing policy makers, health-care providers, and parents. This, in turn, may help formulate policies, and initiate preventive measures and target intervention programs.

Chapter 2

The relation between low-level lead exposure and oxidative stress: a systematic review of epidemiological evidence.

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ABSTRACT:

Experimental studies in animals show evidence that lead exposure results in higher oxidative stress in various tissues in the body, while studies in occupationally-exposed adults also indicate higher oxidative stress markers in workers with high body lead burden. However, this evidence cannot be extended to the general population because of the high levels of lead exposure involved. This systematic review evaluates the epidemiological evidence on the association between lead and oxidative stress at a lower range of exposure with particular attention being given to studies in the pediatric population. Studies were identified through a systematic search of two databases (Medline and Web of Science). The studies included in the review used several biomarkers to assess oxidative stress, which made the comparisons of results across the studies difficult. Studies included in this review do not provide sufficient or convincing evidence that higher body lead burden is associated with higher oxidative stress at a low-level of exposure. Overall, very few studies have been conducted in children and adults from the general population. Most of the reviewed studies suffered from methodological flaws in their design and statistical approach. The findings that were reported are likely to be confounded by many unmeasured or unadjusted factors. Additional well-designed studies with proper statistical methods are needed to establish a causal link between body lead burdens and oxidative stress in the general population.

Keywords: lead, oxidative stress, lipid peroxidation, protein carbonylation, DNA oxidation, DNA damage, children, adults, review.

1. INTRODUCTION:

Although our knowledge of the detrimental effects of lead (Pb) on human health has increased over the years (Needleman, 2004), the mechanism of the metal's toxic actions is still being investigated (Nemsadze, 2009). What is clear is that multiple mechanisms are involved in the various toxic effects of Pb, depending on the target tissue: cellular, intra-cellular and molecular (Needleman, 2004). One mechanism that has received a great deal of attention recently is oxidative stress.

The term "Oxidative Stress" was first defined as "a disturbance in the prooxidant/antioxidant balance in favor of the prooxidants leading to potential damage" (Sies, 1985). Since then, the definition has been refined and more functional outcomes have been included (Jones, 2006). In a more recent definition, oxidative stress is "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" (Sies & Jones, 2007). Under normal physiological conditions, a balance is maintained between endogenous oxidants and enzymatic and non-enzymatic antioxidant systems. When an imbalance occurs, oxidants can cause extensive damage to lipids of cellular membranes, protein and DNA (Halliwell & Gutteridge, 1999; Scandalios, 1997). Oxidative stress has been implicated in the etiology of many diseases and is considered an important pathophysiological process (Dalle-Donne et al., 2006; Elahi et al., 2009; Furukawa et al., 2004; Maritim et al 2003). Animal studies have shown that Pb-induced oxidative stress, at least in part, is responsible for Pb-associated adverse health effects such as hypertension and kidney dysfunction (Dursan et al., 2005; Muntener et al., 2003; Stohs & Bagchi, 1995; Vaziri et al., 2000). Moreover, oxidative stress has been hypothesized as a potential pathway for several other Pb-induced outcomes including pre-eclampsia (Motawei et al., 2013), preterm delivery

(Ahamed et al., 2009), and neurodevelopmental problems (Sanders et al., 2009; Verstraeten et al., 2008). However, there are very few mechanistic studies in humans examining the link between Pb-induced oxidative stress and disease outcomes (Meki et al., 2011). The proposition that oxidative stress plays a critical role in Pb-induced acute and chronic illnesses comes mostly from the evidence in animals and from epidemiological studies showing a relation between body Pb burden and oxidative stress. Because Pb can induce oxidative stress and because oxidative stress has a causative link with most of the health outcomes of which Pb is also a predictor, Pb-induced oxidative stress is a plausible mechanism for many adverse health effects of Pb. Given the high prevalence of low-level environmental lead exposure among adults and children (CDC, 2012), the disease burden attributable to lead is potentially very high. Thus, understanding the extent to which low-level body lead burdens contribute to oxidative stress would close a major gap in knowledge of the health effects of lead in non-occupationally exposed populations.

Putative mechanisms for Pb-induced oxidative stress:

The biological mechanisms behind Pb-induced oxidative stress have been reviewed by Ahamed & colleagues (2007). Briefly, Pb may induce oxidative stress by several ways: inhibiting the enzymes involved in the heme biosynthetic pathway and thereby generating free radicals, increasing the susceptibility of cell-membrane to peroxidation, depleting glutathione (GSH) and changing the activities of the antioxidant enzymes. Lead inhibits the activity of one of the enzymes of the hematopoietic system- δ-aminolevulinic acid dehydratase (δ-ALAD) by binding to sulfhydryl group (-SH) at the active site (thereby replacing zinc), which results in the accumulation of the substrate, δ-aminolevulinic acid (δ-ALA). This is a very unstable compound and can be rapidly converted into free radicals (Gurrer-Orhan et al., 2004). On the cell membrane, Pb may have the ability to change the fatty acid composition of the lipids in a way

that makes the membrane more vulnerable to oxidation (Lawton & Donaldson, 1991). The affinity of Pb towards sulfhydryl groups (-SH) makes GSH more susceptible to binding with Pb, which can result in unavailability of GSH molecule as an antioxidant. Moreover, Pb can alter the activities of antioxidant enzymes by several mechanisms including binding with the replacing and forming complexes with the cofactors required for the functioning of these enzymes. For example, by forming a complex with selenium, Pb can decrease the enzymatic activity of glutathione peroxidase (GPx) (Whanger, 1992). The replacement of Cu and Zn by Pb may decrease the activity of the Cu/Zn-dependent superoxide dismutase (SOD) (Mylorie et al., 1984). While all of these mechanisms are possibly involved in the development or exacerbation of oxidative stress from exposure to Pb, it is not clear whether and which mechanisms operate at different levels of exposure. More importantly, greater understanding of the mechanisms of Pb-induced oxidative stress at a lower level of the body lead burden would inform future studies on the use of biological makers for detecting oxidative stress in the general population. Another aspect of understanding the mechanisms of lead's action is a more accurate assessment of the contribution of lead to disease states that involve oxidative stress. Ultimately, however, the goal is to inform approaches aimed at preventing or alleviating the oxidative damage potentially caused by lead.

Susceptibility to Pb-induced oxidative stress:

Many dietary and genetic factors may increase the vulnerability towards Pb-induced oxidative stress. For example, dietary deficiency of antioxidants (vitamin C, E, tocopherols, carotenoids, selenium) can make individuals more susceptible to the oxidative damage from Pb. Moreover, GSH, the endogenous antioxidant is very susceptible to chelation by Pb because it possesses a sulfhydryl group. As discussed previously, Pb binds to sulfhydryl groups and could

therefore render GSH inactive. Gluthathione reductase (GSR) catalyzes the conversion of oxidized to reduced GSH, thus maintaining adequate intercellular levels of the active form. Optimal functioning of GSR is dependent on the availability of the co-enzyme flavin adenine nucleotide (FAD), which is derived from riboflavin. Riboflavin deficiency can reduce the availability of FAD, thus affecting the amount of reduced GSH, the active antioxidant form (Mulherin et al., 1996).

Apart from dietary intake, polymorphisms of genes encoding the antioxidant enzymes or proteins involved in the uptake and utilization of antioxidants may have an impact on an individual's ability to combat oxidative insults (Da Costa et al., 2012). For example, the polymorphism in the gene *SLC23A1* that encodes the vitamin C transporter (SVCT1) responsible for the transport of vitamin C across epithelial cells of the small intestine (Mandl et al., 2009) or the genes coding for proteins involved in α-tocopherol uptake, transport, and metabolism (such as apolipoproteins, cytochrome P_{450} cholesterol transporter scavenger receptor class B type 1) can affect the amount of these antioxidants in the circulation (Borel et al., 2009). Similarly, gene polymorphisms in antioxidant enzymes like SOD, GPx and CAT may affect the activities of these enzymes (Fabre et al., 2008). Clearly, individual genetic variations and gene-diet interactions may potentially alter an individual's susceptibility to Pb-induced oxidative stress or his ability to counteract any increases in oxidative stress due to lead exposure.

Low-level Pb exposure and oxidative stress:

One criticism of the hypothesis that Pb can induce oxidative stress is that most of the evidence, both in animals and humans, comes from studies of high Pb exposure (Pollack et al., 2012). Our knowledge of the relationship between low-level Pb exposure and oxidative stress is limited. So far, only a few epidemiological studies have tested these associations in the general

population and studies in children are particularly scarce. The ability of Pb to induce oxidative stress in children is important, because it may not only cause adverse effects in childhood but may have long-term effects well into adulthood.

Purpose of the current review:

To advance this field and provide clear research directions, a critical review of the literature is warranted, paying special attention to the question of whether there is enough evidence to support the hypothesis that Pb induces oxidative stress, especially at lower level of exposure overall and in the pediatric population in particular. Previous reviews on this topic have mostly focused on the possible mechanisms by which Pb can cause oxidative stress with the possibility of prevention by antioxidants (Ahamed et al 2007; Hsu & Guo, 2002; Gurer &Ercal 2000; Patrick, 2006). Furthermore, no reviews have been conducted of studies examining lead exposure and oxidative stress in children. The current review is meant to provide an in-depth summary and discussion of the epidemiological studies evaluating the connection between Pb exposure and oxidative stress. In addition, the biological markers for assessing oxidative stress and issues with their measurement are discussed. Finally, future research directions are proposed.

2. METHOD:

2.1. Search strategy:

The aim of the literature search was to identify published articles, both in children and adults, assessing the relation between Pb and oxidative stress. Searches were conducted in Medline and Web of Science from January 1993 through October 2013 by using key words and MeSH terms. Key terms related to Pb exposure and oxidative stress was used to search the Web of Science (**Table 1**). In Medline, combining keywords and MeSH terms were most fruitful in yielding useful results (Table 1). Boolean operators- 'and', 'or', 'not' were used to combine the key words and MeSH terms to retrieve the most relevant articles. The search was limited by species (human), availability of abstract (has abstract) and article type (not a review) in Medline. In Web of Science, the search was restricted by species (human) and document type (article). In addition, the reference lists of all the articles identified through Medline and Web of Science were examined manually to locate additional relevant publications.

Abstracts were only reviewed if the articles were published in English. Studies reporting a wide range of measures of Pb exposure including the biomarkers of Pb in blood, bone and other specimens (such as hair, tooth, nail, urine, semen and placenta), and environmental samples (water, air, soil or dust) were considered for further evaluation of eligibility. No studies using Pb concentrations in environmental samples were identified. Therefore, all studies reported in this review use biomarkers of Pb exposure. The units of measurement for the biomarkers were converted into common units when applicable to facilitate the comparison across studies, for example, blood lead level (BLL) units were converted from mg/l to µg/dL.

Similar to Pb measures, publications with a wide range of oxidative stress measures such as antioxidant enzymes, non-enzymatic antioxidants, end products of oxidative stress (such as

products of lipid peroxidation, DNA oxidation and protein carbonylation), and extent of DNA damage/repair were included for eligibility assessment. The search retrieved *in vitro* studies involving human cell lines, but these were excluded based on a decision *a priori* to only include the *in vivo* epidemiological studies because of their physiological and clinical relevance at the population level. Studies involving multiple metal exposures were only kept if an association between Pb and oxidative stress parameters was examined and reported separately. Studies that evaluated the effects of antioxidants or chelating agents or other compounds on modifying Pb-induced oxidative stress were included if they reported the estimated associations between Pb exposure and oxidative stress markers. Finally, the identified studies included those conducted with occupational workers and the general population.

Table 1: **Search strategy for the review-**

Key words used in Web of Science:

lead exposure, lead toxicity, lead poisoning, lead level, lead concentration, heavy metal, oxidative stress, oxidative damage, lipid peroxidation, protein carbonylation, DNA damage, oxidative DNA damage.

Boolean operators (“and”, “or”, “not”) were used to combine search terms.

Search refined by species (human NOT animal), document type (article) and language (English).

Search term used in Medline:

("lead"[MeSH Terms] OR "lead poisoning"[MeSH Terms]) OR “lead level” OR “lead concentration” OR ("Metals, heavy"[MeSH Terms] NOT (actinium OR americium OR antimony OR barium OR berkelium OR bismuth OR cadmium OR californium OR cesium OR chromium OR cobalt OR copper OR curium OR einsteinium OR fermium OR francis OR gallium OR germanium OR gold OR hafnium OR indium OR iridium OR iron OR lawrencium OR manganese OR mercury OR molybdenum OR neptunium OR nickel OR niobium OR nobelium OR osmium OR palladium OR platinum OR plutonium OR protactinium OR radium OR rhenium OR rhodium OR rubidium OR ruthenium OR silver OR strontium OR tantalum OR technetium OR thallium OR thorium OR Tin OR tungsten OR uranium OR vanadium OR zinc OR zirconium)) AND ("oxidative stress"[MeSH Terms] OR "antioxidants"[MeSH Terms] OR “antioxidant enzyme” OR "lipid peroxidation"[MeSH Terms] OR "DNA damage"[MeSH Terms] OR "protein carbonylation"[MeSH Terms]) AND hasabstract[text] AND "humans"[MeSH Terms] NOT "Review".

3. SUMMARY OF RESULTS:

3.1. Selection of studies:

The search retrieved 191 and 215 references in Medline and Web of Science, respectively. Among these, 15 references in Medline and three in Web of Science were not in English (**Figure 1**). In total, 176 abstracts from Medline and 212 abstracts from Web of Science were screened. In Medline, 9 articles were in non-human species and 47 articles were on studies conducted *in vitro* with human cells. On the other hand, search in web of science yielded 69 articles on non-human species and 95 on studies with human cells. After eliminating the duplicates and reviewing the abstracts, 103 articles were chosen for screening. Articles were not included for screening if the abstracts did not provide any indication of analysis for Pb exposure or biomarkers for oxidative stress. Finally, 76 articles published between 1993 and 2013 met the inclusion criteria and were relevant to the purpose of this review. Among these 76 publications, 16 were conducted in children while 43 included occupationally-exposed workers and 17 environmentally-exposed adults.

3.2. Oxidative stress measurements across the studies:

The biomarkers used to measure oxidative stress, the outcome of interest in this review, varied widely across the studies. In general, there are several assays available for assessing oxidative stress in the body, and consensus regarding the most suitable method for routine clinical assessment of oxidative stress is not available (Jones, 2006). **Table 2** summarizes the currently available biomarkers of oxidative stress and the methods of their assessment. While no available assays measure the balance between pro-oxidants and antioxidants, most focus on measuring either the oxidants and oxidation products or antioxidants and antioxidant systems (Jones, 2006). Concerning the studies related to Pb and oxidative stress, while some measured oxidation products (Cabral et al., 2012; Conterato et al., 2013; Engstrom et al., 2010; Pollack et

al 2012), others assessed concentrations of antioxidants (Diouf et al., 2006; Jin et al., 2006; Prokopowicz et al., 2013) or activities of the antioxidant enzymes (Ahamed et al., 2011; Malekirad et al., 2010; Martinez et al., 2013). Few studies examined the extent of physical damage and/or ability to repair DNA (Jasso-Pinada et al., 2012; Mendez-Gomez et al., 2008; Olewinska et al., 2010) as a consequence of oxidative stress.

Table 2: Biomarkers of oxidative stress

Types of measurements	Biomarkers	Methods of analysis
Oxidants	Superoxide anion, H_2O_2, ROOH, peroxynitrate, other free radicals	Spin trapping, ERS, dROMs
Antioxidants	GSH, Ascorbate or vitamin C, Tocopherols/tocotrienols or vitamin E, Carotenoids, Polyphenols, Trace elements (selenium, zinc)	HPLC, GC-MS
Antioxidant/Pro-oxidant balance	GSH/GSSG redox	HPLC
Antioxidant enzymes	SOD, CAT, GPx, PON, GSR, Trx	Immunoassay, Enzyme activity, mRNA expression
Oxidative damage		
Lipid	Acroleins, Peroxides, MDA, Isoprostanes, CD, HNE, TBARS, HODEs	Immunoassays, TBA*, GC-MS, LC-MS, HPLC
Protein	Carbonylated proteins, Nitrotyrosines	Immunoassays, GC-MS, LC-MS, HPLC
DNA	8-OH-dG, 8-OH-Gua	Immunoassays, GC-MS, LC-MS, HPLC
	Mitochondrial DNA damage	Comet assay, SCE and MN per cell

H_2O_2= hydrogen peroxide; ROOH= hydroperoxides; GSH= Glutathione (reduced form), GSSG= Glutathione disulphide (oxidized form); SOD= Superoxide Dismutase; CAT= Catalase; GPx= Glutathione Peroxidase; PON= Paraoxanase; GSR= Gluthathione Reductase; Trx= Thioredoxin Reductase; MDA= malonaldehyde; CD= conjugated diene; HNE= hydroxyalkenals; TBARS= thiobarbituric acis reactive substances; HODEs= hydroxyl-octadecadienoic acid; 8-OH-dG= 8-hydroxy-deoxyguanosines; 8-OH-Gua= 8-hydroxyguanine; ERS= Electron spin Resonance Spectroscopy; dROMs= derived Reactive Oxygen Metabolites; HPLC= High Performance Liquid Chromatography; GC-MS= Gas Chromatography Mass Spectrometry; LC-MS= Liquid Chromatography Mass Spectrometry, TBA= Thio-Barbituric Acid; SCE= Sister Chromatid Exchange; MN= Micronuclei.

*Test used for assessments of MDA.

The oxidation products assessed in the studies included in this review were the products of lipid peroxidation, protein carbonylation and DNA oxidation. The oxidation (or peroxidation) of lipids was measured as concentrations of peroxides, conjugated diene (CD), malonaldehyde (MDA), 4-hydroxyalkenals (HNE), thiobarbituric acid reactive substances (TBARS), F_2- 8α isoprostane (isoprostane), 9-hydroxy-10,12-octadecadienoic acid (9-HODE), and 13-hydroxy-9,11-octadecadienoic acid (13-HODE). Polyunsaturated fatty acids (PUFA) present in the phospholipid bilayer of biological membranes are particularly susceptible to peroxidation by free radicals (Janero, 1990; Janika et al., 2010). Lipid peroxidation is a complex process and occurs in multiple stages. The products of different stages of lipid peroxidation, for example, primary (peroxides, conjugate diene) or secondary (TBARS, MDA, isoprostane, HNE, HODE etc.) peroxidation products can be measured (Halliwell & Chiodo, 1993). Among these, MDA and isoprostanes are widely used for the purpose of oxidative stress measurements *in vivo*. The concentration of MDA was also a common measure of oxidative stress in many studies described in this review. On the other hand, very few studies assessed the total amount of protein carbonyl content in plasma, a marker for protein oxidation. Carbonyl groups (aldehydes and ketones) are produced on the side chain of amino acids when they are oxidized by free radicals (Dalle-Donne et al., 2006). Finally, DNA is also subject to oxidative attack by free radicals (Ames, 1993; Halliwell, 2000; Loft, 1999). The product of DNA oxidation was measured in the some of the reviewed studies as 8-hydroxy 2' deoxyguanosine (8-OH-dG), which is produced by the hydroxylation of the guanosine moiety of a nucleic acid base by free radicals. 8-OH-dG is excreted in urine and can be assessed by various methods as a general biomarker of oxidative stress (Svecova et al., 2009). Finally, a few studies measured damage to the DNA by the single cell gel electrophoresis also known as comet assay or by assessing sister chromatid exchanges

(SCEs) and the number of micronuclei (MN) per cell. The comet assay allows visualization of the DNA damage and can be applied directly to cells to measure single and double strand breaks in the DNA (Halliwell, 2000; Hartley et al., 2004). SCEs result from reciprocal exchanges of genetic material between sister-chromatids of a chromosome (Latt & Schreck, 1980). The relevance of elevated frequency of SCEs to human health is unknown but their formation seems to be induced by genotoxic agents and believed to indicate DNA damage (Deen et al., 1989). Micronuclei (MN), the small DNA-containing structures, are formed as a result of failure to include the fragments or entire chromosomes in either of the two daughter nuclei during nuclear division. The presence of MN is thought to indicate chromosomal damage (Mielzynska et al., 2006).

Apart from oxidation products, a wide range of antioxidants (endogenous and exogenous) was assessed across the studies reviewed here. The antioxidants measured in these studies included glutathione, tocopherols, vitamin C, carotenoids and selenium (Se). Glutathione, synthesized in the body (by the amino acids cysteine, glycine and glutamic acid), plays a central role as an endogenous antioxidant (Jones, 2006; Sies, 1999; Wu et al., 2004). More than 98% of total glutathione is present as reduced glutathione (GSH) and the rest exists in the oxidized form i.e. glutathione disulphide (GSSG). In the reduced form, the thiol group of the cysteine moiety of glutathione molecule can donate a reducing equivalent which helps neutralize free radicals and reactive oxygen species, maintain thiol/disulfide redox state of proteins, and keep vitamin C and E in their reduced and functional forms (Jones, 2006; Martin & Teismann, 2009; Sies, 1999). Upon donating reducing equivalents, glutathione readily forms oxidized glutathione or GSSG by the seleno-enzyme glutathione peroxidase (GPx), and can be reduced back to GSH by

glutathione reductase (GSR) (Jones, 2006; Wu et al., 2004). A decrease in GSH or an increase in the ratio of GSSG/GSH is considered indicative of oxidative stress.

Tocopherols, vitamin C, carotenoids and selenium are all dietary antioxidants that protect the body cells from oxidative damage. Tocopherols along with tocotrienol compounds are collectively referred to as vitamin E. There are four forms of tocopherols (α, β, ɣ, δ) and among these, α- tocopherol has the highest biological activity (Flohe & Traber, 1999). The antioxidant function of tocopherols or vitamin E is believed to be based in their ability to prevent the propagation of free radical reactions that produce oxidative products and cause damage to the cellular components such as lipids (Burton et al., 1983; Ingold et al., 1987; Packer, 1994; Sandy et al., 1987). Tocopherols are primarily located in cell membranes and exert protective effects against the peroxidation of lipids located in those membranes (Litwack, 2007). Vitamin C, on the other hand, is an important water-soluble antioxidant (Halliwell, 1996). The function of vitamin C as a free radical scavenger or antioxidant is due to its ability to donate electrons, which enables vitamin C to reduce reactive radicals and oxidants (Carr & Frei, 1999; Shills et al., 2006). Vitamin C may also act as a co-antioxidant by regenerating α- tocopherol, glutathione and β-carotene (Carr & Frei, 1999; Halliwell, 1996; Packer, 1997).

Carotenoids, a group of naturally existing pigment compounds, possess the ability to quench ROS (especially singlet molecular oxygen and peroxyl radicals) and protect the cell from oxidative damage (Shills et al., 2006; Stahl & Sies, 2003). Finally, selenium (Se) is an integral component of many antioxidant enzymes including glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) (Tapiero et al., 2003; Tinggi, 2008) and a decrease in Se may affect the activities of these enzymes (Shills et al., 2006).

Studies examining the exposure to Pb and its relation with oxidative stress also assessed the activities of several antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GSR). These enzymes play an important role in protecting cells against oxidative damage by reducing and neutralizing free radicals into less reactive forms (Matés et al., 1999; Prasad, 2011). While SOD reduces highly reactive superoxide anions ($O_2^{\bullet -}$) to less reactive molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), CAT reacts very efficiently with hydrogen peroxide (H_2O_2) forming water and molecular oxygen. The selenium-containing enzyme- GPx catalyses the reduction of hydroperoxides (generated due to lipid peroxidation) and H_2O_2 by using the endogenous antioxidant- glutathione (Matés et al., 1999; Prasad, 2011). On the other hand, GSR reduces oxidized glutathione (GSSG) to its active reduced form (GSH) (Jones, 2006; Wu et al., 2004). Known as the "adaptive response", these enzymes respond to increased oxidative stress by elevating their activities (Prasad, 2011). However, activities may change or decline due to aging (Prasad 2011) or due to binding of sulfhydryl groups by toxins such as arsenic and Pb (Flora, 2009).

In summary, numerous markers of oxidative damage and antioxidant status were evaluated across the reviewed studies. While some studies used multiple markers, others measured only one. Many studies in this review assessed the products of free-radical-induced oxidation of lipids, proteins and DNA as their preferred outcome measures for oxidative stress. This was likely due to a lack of an established standard oxidative stress biomarker and due to the multiple mechanisms involved in the induction and propagation of oxidative stress by Pb, as well as the body's response to elevated oxidative stress levels. Nevertheless, this wide range of measures made the comparison among studies difficult. In addition, any conclusions based on such a range of measures are likely to be tenuous. A systematic measurement of a panel of

biomarkers for both oxidative damage and antioxidant capacity would be helpful in assessing the full dimension of oxidative stress associated with Pb exposure. Since oxidative stress represents the imbalance between oxidative damage and antioxidant repair, a combination of markers for cellular damage and the antioxidant profile would provide a comprehensive assessment of the oxidant/antioxidant balance.

3.2. Overview of studies on Pb and oxidative stress in children-

We identified 16 publications that reported on the associations between the biomarkers of Pb and oxidative stress in children (**Table 3**). Of these, two reports seem to come from the same study conducted in Turkey (Ergurhan-llhan et al., 2008; Oktem et al., 2004). In this study, two groups were compared: one comprised of adolescent boys (15-19 y) working in an auto-repair workshop in the city of Isparta for at least a year with no history of acute or chronic diseases in general, while the controls were healthy boys of the same age-group living in a rural area and with no known history of occupational exposure. The authors assessed BLL as a marker of body Pb burden, whereas oxidative stress parameters included erythrocyte concentrations of MDA, activities of the antioxidant enzymes- GPx, SOD, CAT, and the concentrations of α- tocopherol and β-carotene in the erythrocyte (Ergurhan-llhan et al., 2008; Oktem et al., 2004).

The rest of the studies included in this review examined children from the general population with no known history of occupational exposure. The age of the children across the studies ranged between 0-19 years. Children were healthy, except for two studies, where the children had aplastic anemia (Ahamed et al., 2011) and neurological disorders such as cerebral palsy, seizures and encephalopathy (Ahamed et al., 2008). All these studies in children used blood lead levels (BLLs) as a measurement of body Pb burdens. Children's BLLs varied widely across the studies, with some having BLLs as low as mean (range) 2.6 (0.2 – 28.7) µg/dL

(Martinez et al., 2013) to as high as 28.6 (11.4 – 47.5) µg/dL (Mendez-Gomez et al., 2008). Most of the studies included more than one measure of oxidative stress. Six studies examined the levels of MDA as one of the markers of oxidative stress (Ahamed et al., 2008, 2006, 2005; Cabral et al., 2012; Jin et al., 2006; Oktem et al., 2004). Activities of antioxidant enzymes- SOD, CAT, GPx and GSR were used in seven studies (Ahamed et al., 2011, 2008, 2006, 2005; Diouf et al., 2006; Jin et al., 2006; Martinez et al., 2013), while serum levels of selenium was measured in one study (Diouf et al., 2006). Non-enzymatic antioxidants- GSH and the oxidized/reduced glutathione ratio (GSSG/ GSH) were measured in seven studies (Ahamed et al., 2011, 2008, 2006, 2005; Cabral et al., 2012; Diouf et al 2006; Jin et al 2006). The damage to DNA was assessed in three studies (Jasso-Pinada et al., 2012; Mendez-Gomez et al., 2008; Mielzynska et al., 2006). The associations of body Pb burden in children and these groups of oxidative stress markers are described in detail below.

3.2.1. Children's body lead burden and lipid peroxidation:

Seven studies measured the concentration of lipid peroxidation products. Of these, six assessed the amount of MDA in plasma or erythrocytes (Ahamed et al., 2008, 2006, 2005; Cabral et al., 2012; Ergurhan et al., 2008; Jin et al., 2006) whereas one measured concentration of TBARS as a biomarker for lipid peroxidation (Ahamed et al., 2011). In the study with occupationally- exposed Turkish children, a higher mean erythrocyte concentration of MDA [Mean (SD): 137 (80) vs. 88 (42) nmol/g Hb] and higher BLL [7.9 (5.2) vs. 2.6 (2.0) µg/dL] was observed than in the age-and sex- matched healthy control. A positive correlation was found between children's BLL and MDA in the combined sample [$r = 0.4$; $p < 0.0001$] (Ergurhan-llhan et al., 2008; Oktem et al., 2004). No covariate-adjusted analysis was performed in this study. The controls were selected by matching the age and sex of the occupationally-exposed children and

the authors mentioned no differences in smoking habits or alcohol consumption between the groups. On the other hand, while the controls were attending high school, no information on the educational level of the occupationally-exposed children was reported. In addition, data on other factors such as family's socio-economic status, children's body mass indices (BMI), physical activity, pubertal stage, diet, smoking status, or exposure to other toxicants was not available to evaluate whether differences other than BLL existed among the two groups and whether this could have affected lipid peroxidation. This study indicated that the occupationally-exposed children had significantly higher erythrocyte concentrations of α-tocopherol and β-carotene than the controls (Ergurhan-llhan et al., 2008) and the authors attributed this difference to Pb exposure because they also found a significant correlation between antioxidants and BLL. However, because the analysis was unadjusted and because no dietary data was available, other plausible explanations for the findings may be found.

On the other hand, Cabral and colleagues (2012) conducted a community-based study where they compared Pb and oxidative stress markers among two groups of children (1-16 y old) living in two different areas of Senegal: one near a landfill site and the other 3.5 km away from the landfill. The distance from the landfill for the nearest site was not reported. The landfill consisted of household solids, industrial, hospital, electrical and electronic wastes. Children living near the landfill were possibly engaged in sorting and recovering usable materials from the landfill. Both groups lived in their respective areas since birth, had no history of renal disease and did not consume nephrotoxic substances. Children's BLL [range: 3.25 to 37.34 µg/dL] and urinary Pb levels (U-Pb) [range: 0.02 to 24.3 µg/g creatinine) varied widely, with children living near the landfill having higher BLLs [14.97 (9.70) µg/dL] and U-Pb levels [6.0 (7.7) µg/g creatinine] than those living distant from the landfill [8.22 (3.16) µg/dL and 0.7 (1.2) µg/g

creatinine respectively]. Children living near the landfill had higher MDA concentration when compared to the children who lived at the mre distant site [9.4 (12.2) vs 5.4 (5.9) μM; $p< 0.05$]. The concentrations of metals (such as aluminum, antimony, arsenic, cobalt, cadmium, chromium, copper, iron, lead, manganese, molybdenum, rubidium, strontium, tin and zinc), in air and soil samples from the two residential sites exhibited the presence of multiple metals with higher levels being found in the nearby site. Although the two groups did not differ by age, sex or BMI, no information was available on their socio-economic status (SES), educational levels, family characteristics, health history, and dietary intake or nutritional biomarkers. Differences in all these factors could have contributed to the observed difference in MDA between the groups. Children's BLL and U-Pb were positively correlated with plasma concentrations of MDA in the overall sample [correlation coefficients (r) < 0.4; $p< 0.01$]. However, the analysis was unadjusted and potentially influential covariates were not controlled for. The simple correlation between BLL and MDA does not provide strong evidence for an association between Pb exposure and oxidative stress, particularly causal one.

In another study conducted among 3-12 year olds, the BLLs and oxidative stress markers were compared between children with neurological diseases (cases) and healthy controls (Ahamed et al., 2008). Children with pre-existing neurological disorders like cerebral palsy, seizures and encephalopathy were recruited as cases from a hospital in Lucknow, India. The age- and SES-matched controls were children who lived in and around the city and did not suffer from any chronic conditions (as determined by a physician). The two groups did not differ by age, sex, BMI and socio-economic status but no other information was reported about them. There was a significantly higher BLL [18.6 (7.9) vs. 10.4 (5.1) μg/dL] and higher concentration of MDA in whole blood [20.9 (7.6) vs. 14.9 (5.9) nmol/ml] among cases than controls. In

addition, children's BLL was positively correlated with MDA when two groups were combined [r= 0.37; p< 0.05]. Covariate-adjusted analysis was not performed. Moreover, the study suffered from a lack of reporting on the selection and recruitment of participants, which made it hard to critically assess the appropriateness of the study design. Furthermore, the presence of higher oxidative stress in children with neurological disorder is possible due to various factors such as disease-specific cellular abnormalities, genetic polymorphisms, infection and any illness at birth (Formichi et al., 2009). Consequently, it is difficult to conclude whether the variation in lipid peroxidation between the groups is due to Pb exposure or to the underlying disease conditions.

In contrast, one study was conducted in 3-6 year old Chinese children with no known disease conditions, living in a city with a large steel refinery (Jin et al., 2006). Children were categorized into three groups according to their BLL (< 5, 5 to < 10 and ≥ 10 µg/dL) and concentrations of oxidative stress markers were compared among the groups. No difference in mean concentration of plasma MDA was observed between the lowest (BLL< 5 µg/dL) and the highest BLL group (10 µg/dL). However, when children were stratified at BLL of 10 µg/dL , there was a higher plasma MDA concentration [5.5 (1.1) nmol/mL] in children with BLL ≥ 10 µg/dL than in those with BLL< 10 µg/dL [5.9 (0.8) nmol/mL]. Since children's socio-demographic and biological information was not provided, it is not clear whether the groups differed by any other factors such as age, sex, SES, diet, BMI, physical activity and exposure to other toxicants. Moreover, although statistically significant, the difference in mean plasma MDA concentration between the groups was small and was not derived in covariate-adjusted analyses. Therefore, this study also does not provide strong epidemiological evidence for a link between lead exposure and oxidative stress in children. .

Similarly, Ahamed and colleagues (2006) reported higher blood MDA concentrations [26.3 (5.2) nmol/mL] in 15-18 year old non-occupationally exposed Indian boys with elevated BLL (≥ 10 µg/dL) compared to those with BLLs< 10 µg/dL [17.7 (4.2) nmol/mL]. Children's BLL was positively associated with MDA in an unadjusted regression analysis in that each 1 µg/dL BLL was associated with 1.2 nmol/ml higher concentration of MDA in blood ($p< 0.001$). Notably, the sample size of the study was very small (n= 39) and the authors did not perform any covariate-adjusted analysis. The study children lived in Lucknow, a city with numerous small-scale industries involved in smelting, battery recycling and printing. Since the recruitment procedure was not described adequately, it is not clear how these children were selected. Children were reported as not involved in jobs with potential for Pb or other exposures. However, children's occupational-exposure and medication use were self-reported. There was no significant difference among the groups (BLL< 10 or >10 µg/dL) on age, BMI, types of housing (indicator of socio-economic status), distance of the house from a highway or busy traffic area, and sources of water used in the household for drinking and other purposes. But no information on diet, physical activity or exposure to other toxicants was available. An earlier study by the same group conducted among 4-12 year old children from Lucknow, reported a significantly higher concentration of MDA in whole blood [24.7 (6.8) nmol/mL] in children with BLL> 10 µg/dL than those with BLL≤ 10 µg/dL [14.9 (5.6) nmol/mL] and < 5 µg/dL [16.5 (4.5) nmol/mL] (Ahamed et al., 2005). The difference in MDA between the children with BLL> 10 and ≤ 10 µg/dL was statistically significant but small, and no difference in mean MDA concentration was observed among those with BLL < 10 µg/dL and < 5 µg/dL. A positive correlation [$r= 0.46$; $p< 0.001$] was also observed between BLL and MDA, but no covariate-adjusted analysis was conducted. The authors collected socio-demographical and biological data,

but did not compare the groups on any of the characteristics except for age. As discussed above, many factors including the exposure to other pro-oxidants, antioxidant status or intake, physical activity and BMI could have confounded the relation between Pb and MDA concentration but this was not determined.

Finally, in yet another study, Ahamed and colleagues (2011) examined BLL and the concentration of TBARS in whole blood among 3-12 year old children suffering from aplastic anemia compared to healthy controls. A higher mean concentration of BLL [9.86 (2.06) vs. 4.23 (1.23) µg/dL] and TBARS [23.3 (4.0) vs. 13.3 (3.9) nmol/mL] was found in children who had aplastic anemia than in the control group A positive correlation between BLL and the concentration of TBARS [r= 0.41; p< 0.05] was also found when the entire sample was studied together. No covariate-adjusted analysis was performed. In this study, cases were diagnosed with aplastic anemia and recruited from a pediatric hospital in Lucknow. The controls were children with normal blood counts who did not suffer from any chronic condition. They were matched on age, sex and socio-economic status. However, the recruitment of the controls was not adequately described to make an assessment of their appropriateness. The two groups did not differ by BMI and the area of living (rural vs urban). However, no other information was available nor comparisons made between the two groups. Aplastic anemia is a blood disorder where the bone marrow does not make enough red blood cells (RBCs). Apart from genetic influences, the known risk factors for aplastic anemia, particularly for the type acquired postnatally include exposure to arsenic, benzene and pesticides (Schrezenmier &Bacigalupo, 2000). Therefore, there may be several plausible biological, environmental and genetic factors other than Pb that could contribute to higher oxidative stress observed in the children with aplastic anemia.

In summary, although biomarkers of lipid peroxidation occurred in higher concentrations among children with higher BLL compared to those with lower BLL, and a positive correlation was consistently observed across the studies, the studies suffered from several design and analytical issues that limit the conclusions that can be drawn from them. For example, sample selection is questionable partly due to sparse reporting and partly due to the existence of actual problems in the selection procedure. Recruitment criteria were not adequately discussed in many of the publications. Adequate data on socio-demographic, biological, dietary or environmental factors were not collected or reported. Furthermore, the sample sizes and the effect sizes in most of the studies were small. Co-exposure to other toxicants capable of inducing lipid peroxidation was highly plausible and without proper adjustment, the independent relation between Pb and lipid peroxidation cannot be claimed. Finally, in children with existing disease conditions, the contribution of the disease rather than Pb exposure towards lipid peroxidation was not considered. Well-designed studies with adequate sample size, proper assessment and adjustments of the confounding factors along with detailed reporting are warranted.

3.2.2. Body lead burden and antioxidant status:

Enzymatic and non-enzymatic antioxidants were measured in 10 studies conducted among children. GSH or GSSG/GSH was measured to assess children's oxidative stress in seven studies. A lower GSH concentration or higher GSSG/GSH is generally considered an indication of higher oxidative stress (Jones, 2006). A significantly lower blood GSH concentration was observed in children suffering from neurological conditions [16.2 (3.9) vs. 24.9 (5.2) µmol/mL] (Ahamed et al., 2011) or aplastic anemia [17.9 (5.5) vs. 22.2 (6.3) µmol/mL] (Ahamed et al., 2008) who also had higher mean BLL, than among the control groups who also had lower BLLs. An inverse correlation was also observed between BLL and GSH in the overall samples [r= -0.4 (Ahamed et al., 2011), -0.31 (Ahamed et al., 2008); $p< 0.05$]. No covariate-adjusted analysis was

performed. These studies have been described in detail previously, and as discussed before, various factors related to the underlying disease conditions could have affected the oxidant-antioxidant balance in the body. Moreover, the blood concentration of GSH could be low in a blood disorder like aplastic anemia due to the low RBC production by bone marrow. In their 2005 study in non-occupationally exposed Indian children with no known disease conditions, the authors reported lower blood GSH concentrations [13.7 (4.9) µmol/mL] in children with BLL> 10 µg/dL than those with BLL 5 to 10 µg/dL [23.2 (6.5)]. An inverse correlation was observed between BLL and GSH [r= -0.6; p< 0.001], but no covariate adjustments were conducted (Ahamed et al., 2005). No information on the exposure to other toxicants with the potential for reducing GSH levels, other antioxidant status or intake, physical activity or BMI was available.

In contrast, two other studies in non-occupationally exposed healthy children did not find any significant difference in GSH when comparing between children with BLL< 10 and ≥ 10 µg/dL (Ahamed et al., 2006; Jin et al., 2006). Other studies found conflicting results regarding BLL and GSSG/GSH. While a study by Cabral and colleagues (2012) compared BLLs and GSSG/GSH in 1-16 year old children living nearby or away from a major landfill in Senegal, Diuof and colleagues (2006) chose two groups of children (aged 8-12 years), one living in Dakar, Sengal and the other 100 km away from the city. Cabral and colleagues (2012) found higher BLLs in children living near the landfill than those living at the distant site [14.97 (9.70) vs. 8.22 (3.16) µg/dL], but no difference in GSSG/GSH between the two groups [3.3 (2.5) vs 3.1 (2.3), respectively]. On the other hand, in the study by Cabral and colleagues (2006), children from the urban site had higher mean BLL [9.9 (3.9) vs. 5.2 (5.9) µg/dL] and higher GSSG/GSH [1.7 (3.5) vs. 1.01 (3.1)] than those in the rural site. Neither study found any correlation between BLL and glutathione status, and no covariate-adjusted analyses were performed. Children in both

the studies were included if they made the age criterion, were residents of the chosen areas since birth and had no history of chronic conditions. While the groups did not differ by age, sex or BMI, no information was available on their socio-economic status, educational levels, family characteristics, health history, and dietary intake or nutritional biomarkers (Cabral et al., 2012; Diouf et al., 2006). The group difference in glutathione status found in one study but not in other could not be easily explained because little additional information was available. It is possible taht differences in the choice of study and comparison groups in these two studies contribute to the conflicting findings. Children in the study by Cabral and colleagues (2012) had multiple metal exposures, whereas the exposure to other toxicants is unknown for children in the study by Diouf and colleagues (2006). The production of reduced GSH from oxidized GSSG is dependent on the selenium-containing enzyme Glutathione Reductase (GSR). Diouf and colleagues (2006) did not find any relation between BLL and GSR in their study. The authors also compared blood selenium concentration between the two groups in unadjusted analyses and found lower concentration of Se in children in the urban area [64.2 (18.6) vs. 103.3 (33.5) µg/dL] than those at the rural site. No correlation was observed between children's BLL and Se concentration (Diouf et al 2006). As with other antioxidants, variation in blood Se could be due to several factors, including differences in the dietary intake. Since no dietary data was available for these children, no explanation can be provided.

In another study of occupationally-exposed Turkish adolescents with higher BLL, plasma levels of α- tocopherol and β- carotene were lower in workers [α- tocopherol: 9.0 (4.0) ppm; β- carotene: 16 (15) ppb] than the non-occupationally-exposed controls with lower BLL [α- toccopherol: 13 (7) ppm; β- carotene: 27 (23) ppb] (Ergurhan-llhan et al., 2008). The study reported negative correlations between children's BLL and plasma levels of α- tocopherol

[r= -0.5; p< 0.01] and β- carotene [r= -0.6; p< 0.01] in the overall sample. As discussed previously for this study, no information on dietary intake of these two groups was available. This is also true for socio-demographic, biological and environmental factors that could potentially explain the reported group differences

In regard to assessments of antioxidant enzyme activities, the studies represent a mix of those examining just one and those assessing multiple enzymes. Two biological considerations need to be taken into account while interpreting the results of antioxidant enzyme activities. The activities of these enzymes normally increase as the body responds to an increase in free radical production (Dalle-Donne et al., 2006; Rahman, 2007). Alternatively, a decline in enzyme activity is possible due to the ability of pro-oxidants such as Pb to replace and form complexes with enzymatic cofactors (such as Cu, Zn, Se), thus altering enzymatic function (Ahamed & Siddiqui, 2007). When enzymatic activity is assessed in cross-sectional studies, where the timing and duration of exposure is uncertain, the interpretation of the findings is made particularly difficult by this duality of response in the antioxidant defense system.

Martinez & colleagues (2013) assessed the activities of SOD and CAT in 0-14 year old Argentinian children who had a mean (SD) BLL of 2.6 (0.3) µg/dL. Children were selected randomly from among those attended by a hospital in the city of Cordoba and had minor health problems (such as headaches, colds, abdominal pain etc.) or for routine check-ups. Exposure to other toxicants, dietary intakes or other biological and demographic characteristics was not reported. There was no significant correlation between BLL and SOD or CAT activity in the unadjusted analyses that were presented.

In contrast, the two case-control studies in children with neurological disorders or aplastic anemia that had been described previously compared the cases to healthy controls, and

reported higher CAT and SOD activities among the cases (Ahamed et al., 2011, 2008). Children with aplastic anemia had higher BLL and exhibited higher CAT activity [119.7 (15.3) vs. 82.3 (8.7) µmol H_2O_2 decomposed/min/g Hb] than the controls. As before, no covariate-adjusted analysis was performed. Similarly, cases with neurological conditions had higher BLL, and higher CAT [102.2 (13.5) vs. 87.7 (19.7) µmol H_2O_2 decomposed/min/g Hb] and SOD [4.4 (1.3) vs. 1.8 (0.7) nmol epinephrine oxidized/min/g Hb] activity than the controls. Moreover, in two very similar studies, Ahamed & colleagues reported higher CAT activity in non-occupationally-exposed children with BLL> 10 µg/dL [75.4 (11.3) µmol H_2O_2 decomposed/min/g Hb, (2006); 94.7 (17.3) µmol H_2O_2 decomposed/min/g Hb, (2005)] than children with BLL≤ 10 µg/dL [65.1 (10.1) µmol H_2O_2 decomposed/min/g Hb, (2006); 81.4 (24.3) µmol H_2O_2 decomposed/min/g Hb, (2005)]. Jin & colleagues (2006) did not find significant differences in the activities of SOD and GPx when comparing children with BLL> 10 µg/dL to those with BLL≤ 10 µg/dL. In unadjusted analyses, positive correlations of BLL with the activity of SOD [r= 0.53; p< 0.05] (Ahamed et al., 2008) and BLL with the activity of CAT [r: 0.3 - 0.5; p< 0.05] were also reported in studies by Ahamed & colleagues (2011; 2008; 2006; 2005). Appropriate covariate-adjustment was not conducted in any of the studies. Similarly, in occupationally-exposed Turkish adolescents, higher GPx activity [37 (9) U/g Hb] was reported in workers than in controls [30 (9) U/g Hb] (Ergurhan-llhan et al., 2008). Positive correlations were found between BLL and CAT [r= 0.7; p< 0.05] and BLL and GPx [r= 0.2; p< 0.05] activities when the groups were combined (Ergurhan-llhan et al., 2008). In contrast, a study in Senegalese children from rural and urban sites with no known history of occupational exposure found a negative correlation between BLL and GPx activity [r= -0.2; p< 0.01]. Covariate-adjusted analysis was not performed to confirm the findings (Diouf et al., 2006).

In summary, studies assessing BLL and antioxidant status (enzymatic and non-enzymatic) found lower concentrations of antioxidants and higher activities of antioxidant enzymes in children with higher versus lower BLL. Although fairly consistent, this evidence is based on statistical analyses that did not account for other factors that could influence antioxidant status, and should therefore be interpreted with caution. Many dietary, genetic, physiological and environmental factors play an important role in the body's antioxidant response, but no study took these into account Furthermore, to strengthen the evidence base, the type of antioxidants to be evaluated in studies needs to be more consistent. Finally, oxidative stress is an imbalance between oxidants and antioxidants; an evaluation of antioxidant concentrations or antioxidant enzyme activities without an assessment of oxidant biomarkers does not provide a complete evaluation of the oxidative stress being experienced.

3.2.3. Body Pb burden and DNA damage in children:

Findings from studies that measured the relation between BLL and DNA damage in children are inconsistent. This is further complicated by the fact that only three studies to date have conducted such assessments and did so using different methodologies. One study assessed the physical damage to DNA as numbers of micronuclei (MN) and sister-chromatid exchange (SCE) in peripheral lymphocytes in 5-14 years old Polish children (Mielzynska et al., 2006). Children were recruited from two cities (Katowice and Sosnowiec) in Poland with known history of Pb exposure and air pollution. Apart from BLL, the authors measured biomarkers for polycyclic hydrocarbon (PAH) exposure (urinary levels of 1-hydroxypyrene or 1-OHP and PAH-DNA adducts). Detailed information on family child socio-demographic characteristics, family and child health history, medication use, x-ray exposure and dietary habits (fruits and vegetable consumption) were collected from the parents via a questionnaire. Children's mean (SD) BLL

was 7.7 (4.3) µg/dL and ranged from 2.7 to 23.0 µg/dL. When comparing the differences in DNA damage, a significantly higher MN was observed in children wtih BLL> 10 than those with BLL≤ 10 µg/dL [6.4 vs 3.9 MN cells/10^3 binucleated cells $p< 0.05$]. Moreover, in a covariate-adjusted regression model, there was a strong positive association of BLL with the number of MN per 1000 cells [regression coefficient (β) = 0.4; $p< 0.01$], but no association with SCE. The analyses were adjusted for age, sex, exposure to environmental tobacco smoke (determined based on cohabitation with smokers), indoor emission from coal-burning stoves (measured by information on the type of heating system in the household) and parental education. The multivariate regression model also included levels of 1-OHP and PAH-DNA adducts, biomarkers for PAH exposure. It is important to note that although this is one of the few studies on Pb exposure and oxidative stress that collected dietary information, the article did not report any diet-related information and did not adjust for dietary factors in the analysis (Mielzynska et al., 2006). Nonetheless, this study stands out as one of the few that adjusted for potential confounding factors, thus moving beyond evidence based on simple correlations.

Conversely, two studies in Mexican children did not find any significant association between children's BLL and DNA damage in multivariate analyses (Jasso-Pinada et al., 2012; Mendez-Gomez et al., 2008). These two studies measured DNA damage by single-cell gel electrophoresis or comet assay, followed by an assessment of the cell's ability to repair H_2O_2-induced DNA damage. Jasso-Pinada & colleagues (2012) recruited children from three areas of Mexico: one at a mining site (Villa de la Paz) (no information on types of minerals found in the site), the second 8 km away from Villa de la Paz (with a copper smelter that also produced Pb), and the third an agricultural area 200 km away from Villa de la Paz. This was a school-based study of children attending first to sixth grades. Children's geometric mean (SD) BLL in three

communities were 11.4 (0.6), 7.3 (1.5), 5.3 (0.5) µg/dL, whereas and urinary arsenic (UAs) concentrations were 44.5 (4.8), 16.8 (1.6) and 12.8 (1.3) µg/gc, respectively. In unadjusted analysis, the authors observed higher DNA damage (higher tail moment found in comet assay) in children living at the mining site with highest BLL than children at the other sites. In a multivariate regression model, that also included predictors such as age, sex, height and nutritional status, no associations were found between BLL or UAs and DNA damage. The authors of this study did not define nutritional status and it is difficult to speculate whether they included children's weight or BMI as an indicator of nutritional status in the final model. On the other hand, Mendez-Gomez & colleagues (2008) conducted a study in children attending primary schools located at different distances from a smelter complex in Torreon, Mexico, that produced Pb, As and other metals. One group of children attended a school 650 m away from the smelter, whereas the other two groups went to schools that were 1750 m and 8100 m away from the smelter. Children who attended the school nearest to the smelter complex had higher BLL [28.6 (11.4 – 47.5) µg/dL] than those who went to the more distant schools [19.5 (11.3 – 49.2) and 4.6 (0.1 – 8.7) µg/dL, respectively]. Similar patterns were observed for children's UAs or metal concentrations in environmental samples (dust and water) collected from the school-sites. Children attending the school nearest to the smelter had higher DNA damage (evaluated by comet assay) than those whose schools were located further away. However, no association between BLL and DNA damage was found in multivariate regression analysis with the overall sample after controlling for age, sex, self-reported use of Pb glazed pottery by the families and children's UAs concentration.

In summary, there are very few studies assessing DNA damage as a marker of oxidative stress in lead-exposed children. The studies included in the review represented methodological

differences in the assessment of DNA damage, as well as in the factors that were included in the multivariate regression models. These differences make the comparison of findings difficult. Only one study found a statistically significant association between BLL and DNA damage in children even after adjusting for confounders. However, the sample size of the study was small (n= 74) and measurements for antioxidant status or dietary intakes were not included in the analysis. Additional studies are needed to investigate potential Pb-induced DNA damage.

3.3. Summary of findings from the studies in children:

Majority of the studies in children measured BLL as a biomarker of body Pb burden and lipid peroxidation and antioxidant concentrations or antioxidant enzyme activity as a measure/s of oxidative stress. Very few studies assessed DNA damage. Studies varied in their sample selection, but most recruited children in such a way that comparisons in oxidative stress markers could be made between groups of children with higher BLL (workers, children with anemia and neurological disorders, children living near the source of exposure or living in urban areas) and lower BLL (non-occupationally exposed healthy children or those living in rural areas). Mean BLLs for children in higher vs lower exposure groups varied across the studies. Few studies categorized children according to the previous reference level for BLL in children set by CDC (10 µg/dL) and compared the oxidative stress markers between these categories. Studies consistently found higher concentrations of the products of lipid peroxidation (such as MDA and TBARS) in children with higher BLLs. All studies that measured MDA also found positive correlations between BLL and MDA concentrations but none performed covariate-adjusted analyses to further investigate these associations. Few studies observed lower GSH concentrations in children with higher BLL but reported lower GSH concentrations with higher BLLs. Studies assessing the activities of antioxidant enzymes found mixed results. However, as discussed before, caution should be taken while interpreting these results because they are based

on unadjusted analyses. Most of the studies did not collect and/or report important information on socio-demographic, physiological, dietary or environmental factors and provided sparse information on sample selection. Particularly for case-control studies, the process for selecting cases and controls is critical because the controls need to derive from the same underlying population to reduce bias. It is unclear how closely some of the studies adhered to this procedure. Studies of DNA damage due to lead exposure were covariate-adjusted and therefore represent stronger epidemiological evidence, but only one study reported a positive association between BLL and DNA damage. More studies are needed to elucidate the association between lead exposure and oxidative stress in children, and particular attention needs to be paid to potential confounding factors. Unfortunately, the current evidence base includes a dearth of well-designed studies with adequate sample size and proper statistical analyses.

3.4. Relation between body lead burden and oxidative stress in adults

3.4.1. Summary of studies in general adult population:

Although the primary focus of this review is the evaluation of studies related to Pb exposure and oxidative stress in children, we also identified and summarize studies conducted in adults. We identified only five studies that examined the relation between biomarkers of Pb exposure and oxidative stress in environmentally-exposed adults (**Table 4**). Four out of five studies were conducted in women, with three of them during pregnancy. In a sample of healthy premenopausal, non-pregnant US women, no association was found between BLL [Median (interquartile range) 0.86 (0.58 - 2.0 µg/dL] and lipid peroxidation products (isoprostane, 9-HODE, 13-HODE and TBARS) after adjusting for age, BMI, smoking status and race (Pollack et al 2012). In contrast, Lee and colleagues (2006) examined the relation between BLL and antioxidants in non-pregnant US adults including men, aged ≥ 20 years, participating in the

NHANES III. After adjusting for age, race, sex and socio-economic status, higher BLL [Geometric mean (range): 2.8 (0.7 – 56 µg/dL) was associated with higher concentrations of serum γ-glutamyl transferase (GGT), an antioxidant enzyme (Lee et al 2004). In addition, BLL was inversely associated with serum levels of non-enzymatic anti-oxidants like vitamin C, carotenoids and vitamin E (Lee et al 2006) (Table 4). In Bangladeshi pregnant women exposed to high levels of As in water, Engstrom and colleagues (2010) did not find any association between BLL or UPb and urinary concentrations of (8-OHdG, the product of DNA oxidation. The analysis was adjusted for age, gestational week, plasma ferritin and concentrations of zinc, selenium and manganese in blood. In this study, UAs and urinary cadmium (UCd) concentrations were statistically associated with 8-OHdG, suggesting that in the presence of high levels of pro-oxidants such as As and Cd, the contribution of Pb towards oxidative stress may be small or even undetectable.

On the other hand, Serafim and colleagues (2012) examined the relation between placental Pb concentrations and lipid peroxidation in placental tissues of 17-40 year old pregnant women living in South Portugal. Lipid peroxidation (LPO) was expressed as the total concentration of MDA and 4-hydroxyalkenals (4-HNE) per gram of protein. Higher placental Pb concentration was positively correlated with LPO (Serafim et al 2012). The pregnant women in this study were exposed to other toxic metals such as cadmium (Cd), nickel (Ni) and chromium (Cr). However, the analyses were not adjusted for the concentration of these metals or for any other socio-demographic, biologic or dietary factors. In 20 to 35 year old Indian pregnant women, placental Pb concentrations were positively correlated with plasma TBARS concentrations and the activities of antioxidant enzymes SOD and CAT (Ahamed et al., 2009). Moreover, placental Pb concentrations were negatively correlated with the concentration of GSH

in placenta (Ahamed et al., 2009). The authors attributed the lower levels of the endogenous antioxidant GSH with higher BLL to the role of GSH. On the other hand, the higher activities of the enzymes SOD and CAT with higher BLL were thought as an accelerated effort of the anti-oxidant defense system of the placenta to protect against Pb-induced free radicals (Ahamed et al., 2009). No relation was reported between placental Pb concentrations and the activities of the enzymes GPx, GSR or glutathione-S-transferase (GST) (Ahamed et al 2009). Because the primary objective of the study was to compare placental Pb concentration and oxidative stress markers among women with pre-term and full-term deliveries, women were recruited based on gestational age at delivery (Ahamed et al., 2009). No adjustment for pre-term delivery, existing health conditions, nutritional status or any other factors was carried out in this study.

In summary, apart from two studies (Lee et al., 2004; Pollack et al., 2012), most of the work on oxidative stress in non-occupationally exposed adults was done in pregnant women. Pregnancy is a condition when the susceptibility to oxidative stress increases mostly due to increased production of free radicals in the mitochondria-rich placental tissues that supply oxygen and important nutrients to the fetus (Casanueva & Viteri, 2003). The increase in oxidative stress in pregnancy also varies by gestational age, with highest production of free radicals observed in the second trimester (Casanueva & Viteri, 2003). Except for the study by Engstrom and colleagues, none of the studies adjusted for gestational age in their analysis. Consequently, evidence from the studies in pregnant women cannot be extended to the general population. Furthermore, because only two studies were conducted in the general population with low-level environmental exposure to Pb, it is not possible to make any firm conclusions about the relation between body Pb burden and oxidative stress in adults.

3.4.2. Studies in occupationally exposed adults-

We identified 17 studies that examined the relation between Pb exposure and oxidative stress in occupationally-exposed adults (Table 4). In general, the BLLs of the occupationally exposed workers were higher than that observed in the general population. The majority of the studies compared Pb concentrations and oxidative stress markers of industrial workers to those involved in office-based or administrative jobs. Among the 17 studies in occupationally exposed workers, 13 measured the products of lipid peroxidation, including serum/plasma or erythrocyte concentrations of MDA, plasma total peroxides, CD, and the rate of LPO (Table 4). Most of the studies reported higher lipid peroxidation in workers with higher BLL compared to the non-occupationally-exposed controls. Moreover, studies that examined the relation between BLL and lipid peroxidation markers mostly found significant positive correlation between the two measures (Table 4). Only one study in Brazilian workers and non-exposed controls, with BLL ranging from 0.4 to 89.7 µg/dL, reported no correlation between BLL and plasma concentrations of MDA in the overall sample (Conterato et al., 2011). However, there was a positive correlation between BLL and the activities of the anti-oxidant enzymes GPx, SOD and GST, but not with others [e.g. CAT and Thioredoxin reductase (TrxR)]. Some studies (e.g. Gurer-orhan et al., 2004; Kasperczyk et al., 2012; Khan et al., 2008; Malekirad et al., 2010) found higher activities of anti-oxidant enzymes in occupationally exposed workers than in controls, while others (Grover et al., 2010, Mohammed et al., 2008, Patil et al., 2006) reported lower anti-oxidant enzyme activities in workers than the controls (Table 4). None of these studies controlled for other environmental, nutritional or socio-demographical factors.

Studies that tested the extent of DNA damage in occupationally Pb-exposed workers, mostly found higher DNA damage in workers than in the controls (Grover et al., 2010;

Malekirad et al., 2010; Olewińska et al., 2010; Ye et al., 1999). When the relation between body Pb burdens and DNA damage was examined, few studies found significant positive correlations (Costa et al 1997; Grover et al., 2010; Olewińska et al., 2010, Ye et al., 1999), while others reported null findings (Garcon et al., 2007; Malekirad et al., 2010). Lin and colleagues (2012) employed a study design without a non-occupationally exposed comparison group. The study was conducted in Taiwanese workers engaged in different jobs in a glass factory and these workers experienced varying levels of Pb exposure. There was no association between workers' U-Pb and 8-OHdG in multivariate regression analysis, adjusted for age, smoking behavior, alcohol consumption, exposure to heat, dust and other elements (measured as urinary levels of As, Cd, Ni, and Se). Although the statistical models were adjusted for potential confounders, the use of U-Pb as a marker of Pb exposure is limiting because of uncertain relationship between U-Pb and BLL, and U-Pb and other functional outcomes. In adults, Pb is deposited in the bones and can remain in those deposits for many years, with some excretion occurring through the kidney (Barbosa et al., 2005).

In another study, Garcon and colleagues (2004) found positive correlations of BLL with plasma MDA concentrations [r= 0.4; p< 0.01] and SOD activity [r= 0.2; p< 0.05], and an inverse correlation between BLL and serum Se concentration [r= -0.2; p< 0.05] in workers from a metal smelter in France.U-Pb and urinary 8-OHdG concentrations were uncorrelated (Garcon et al 2004). No covariate-adjusted analyses were reported.

In summary, the preponderance of evidence on the relation between Pb exposure and oxidative stress in adults comes from occupationally-exposed workers. Although, the evidence may suggest that higher BLLs are associated with higher oxidative stress in workers, some issues regarding participant selection and analytical approaches should be considered. These issues

represent important study design limitations that affect the interpretation and generalization of the findings, and are very reminiscent of the issues discussed for the studies in children. First, most of the studies did not compare the socio-demographic, life style, dietary or other characteristics of occupationally-exposed workers with non-occupationally-exposed controls to understand whether these differed and could therefore potentially influence the findings. Second, the studies did not control for exposures to other toxicants in their analysis, which is highly plausible in occupationally-exposed populations, and could actually drive the observed associations. As was shown by Engstrom and colleagues (2010) in non-occupationally exposed individuals, it was actually the concentrations of As and Cd that were more salient predictors of oxidative stress than Pb exposure. In industrial workers, where exposure to metals is higher than in the general population, these relationships among metals are likely to be even more pronounced. Finally, in adults both long-term and current exposures are important. The use of BLL (measurement of current exposure) or UPb (can reflect long-term exposure but generally not considered a sensitive marker of Pb exposure) (Barbosa et al., 2005) may only partly reflect the true burden of oxidative stress experienced by an individual over his/her working life, particularly given the considerations of how antioxidant enzyme systems respond to oxidative stress in the short and long-term. Studies measuring lead in bone would be useful to estimate the relationship between chronic high Pb exposure and oxidative stress.

4. DISCUSSION:

The primary purpose of this literature review was to summarize the epidemiological evidence on the association of Pb exposure with oxidative stress, focusing on low-level Pb exposure, particularly in children. Overall, the review suggests that the current evidence from epidemiological studies is not sufficient to infer that higher body Pb burden is strongly or causally associated with oxidative stress. Evidence for a dose-response relation between low level Pb exposure and oxidative stress is rare. The studies that found higher oxidative stress markers in those with higher vs lower body Pb burdens or the correlation between Pb and oxidative stress is seriously limited by several methodological problems, and the current evidence would be based almost entirely on case-control or cross-sectional studies. Prospective cohort studies provide a higher burden of proof for causal relationships but have thus far not been employed to investigate these questions. Randomized controlled trials (RCTs) are not ethical, at least if considering randomization to exposure. RCTs of interventions to lower oxidative stress in occupationally or environmentally exposed populations are possible but premature given the current state of evidence on the association between Pb exposure and oxidative stress. Additional studies with better design and statistical methods are highly recommended to establish the extent of the relation between Pb exposure and oxidative stress. These issues are discussed in detail below.

4.1. Limitations of study designs:

All the epidemiological studies assessing the relation between body Pb burden and oxidative stress are observational in design. Although experimental design is the strongest study design for drawing causal inferences regarding relations between exposure and health outcomes, it is not always practically or ethically possible to conduct such experiments in humans

particularly in toxicological research (Checkoway et al., 2004). Moreover, in toxicology, it is important to assess how exposure to toxicants affects human health over decades of chronic exposure. For this, observational studies in humans are extremely valuable (Checkoway et al., 2004) and provide important evidence along with experiments in animals to guide public health policies. However, in observational studies, choosing appropriate designs and analytical procedures is critically important for understanding the relationship between an exposure and an outcome (Hatch & Thomas, 1993). One of the limitations of the studies reviewed here is their approach to compare the concentration of oxidative stress markers among participants with higher vs lower body Pb burden, without considering the continuity in the range of exposures. It is particularly important to understand the relationship between Pb and oxidative stress within a range of body Pb burdens, because Pb has been associated with adverse health outcomes at very low levels of exposure with no evidence for a safe threshold (Bellinger, 2011). Where correlations were carried out, it was typically on a combined sample of cases and controls, which is a limitation particularly because in some of the studies the two groups likely did not represent the same underlying population group.

Another important limitation is that most of the reviewed studies only performed unadjusted analyses. Adequate information was not provided to make critical assessment as to whether any difference other than body Pb burden existed between the groups or affected the study outcomes. While correlations or unadjusted regression models can provide a preliminary understanding of the strength or direction of the relation between Pb levels and oxidative stress, the observed associations may be confounded by many other factors not accounted for in the unadjusted analyses. For example, factors that may potentially distort the observed association between Pb exposure and oxidative stress include exposure to other toxicants (such as As, Cd,

Ni, Hg and PAH) (Ercal et al., 2001; Flora, 2009; Kuang et al., 2013; Stohs & Bagchi, 1995), smoking (including second-hand smoke) (Chalmers, 1999; Isik et al., 2007), age (Block, 2002; Sanchez et al., 2005), body mass index (Higdon & Frei, 2003; Wonisch et al., 2012), physical activity (Jenkins, 2000; Leaf et al., 1999), individual genetic variations (De Costa et al., 2012; Fabre, 2008) and dietary factors (Block, 2002; De Costa et al., 2012). Only a few studies controlled for some of these factors in their statistical analyses (Engstrom et al., 2010; Li et al., 2006; Pollack et al., 2012). In addition, where adjustments were made, they assumed confounding or mediation. Conversely, the above factors could actually act as effect-modifiers that could modify the strength of the association between body Pb burden and oxidative stress, a hypothesis that has so far not been tested in epidemiological studies.

Another limitation of the study design is the measurement of both the biological markers for Pb and oxidative stress at one point of time. Detectable differences in oxidative imbalance, on the other hand, could take years to develop, since the antioxidant defense system may be initially able to fight off the effects of free radicals produced by Pb, particularly when the exposure is low. A prospective cohort study with repeated measures would be a more appropriate design to detect oxidative stress and thus strengthen the evidence for a potential cause-effect relationship between Pb exposure and oxidative stress.

Further, generalizability of the findings needs to be taken into consideration. Most of the studies in non-occupationally exposed children recruited participants who lived near a major source of exposure such as mines, landfills, refineries or smelters, thus the exposure levels to many toxicants including Pb were higher than for children living away such sources. Children living near a source may differ not only in their own current exposure status, but on many other factors, including prenatal exposure. It is possible that these children are actually more

susceptible to oxidative stress because their systems have been "primed" by their mothers' exposure to various toxicants during pregnancy or due to lower socio-economic status and poor diet quality.

Similarly, the majority of studies in adults were conducted in occupationally-exposed workers with high body lead burdens not typically observed in the general population. The findings derived from these studies cannot be easily extended to the general population because of this fact, and because many other factors including exposure to other toxins, smoke, heat, occupational hazards and related stresses, life-style factors such as smoking, alcohol consumption, dietary intake, physical activity, BMI and socio-economic status are likely to differ among workers and the general population.

Finally, the studies in both adults and children measured a variety of biomarkers of oxidative stress, which has many advantages including increasing the chances for detecting oxidative stress and understanding the nature of the damage that is occurring in the body (ie., tissues or physiological systems being affected). However, some consistency in the use of biomarkers across the studies would help in interpreting and comparing the studied and drawing clearer conclusions. In addition, careful considerations should be given to collect data on the factors other than Pb that could potentially affect these biomarkers, such as exposure to other environmental toxins, dietary and lifestyle factors (such as smoking, alcohol consumption etc.), physiological conditions (pregnancy, aging), body weight, physical activity, and individual genetic variations in the antioxidant defense system and adequately control them in the analysis with proper statistical methods, a process that was largely missing in the studies reviewed here.

4.2. Is there enough epidemiological evidence for the association between higher body Pb burden and oxidative stress?

The design limitations discussed above clearly suggest that not enough clear evidence is currently available to support the hypothesis that higher body Pb burden is associated with increased oxidative stress in general population, particularly in a range of exposure currently observed around the world. Studies in children are not adequately designed and/or the statistical approach is not appropriate to clearly discern the relation between Pb exposure and oxidative stress at lower ranges of exposure. Environmentally-exposed children in most countries now suffer from chronic low-level Pb exposure, with mean BLL being <10 µg/dL (Attina & Trasande, 2013). Well-designed studies with appropriate measurements and control for confounders are needed to answer these questions.

On the other hand, studies in adults were mostly conducted in occupationally-exposed workers and suggest that the workers with higher Pb exposure do experience higher oxidative stress than those not involved in factory work. But as mentioned before, caution should be taken when interpreting the results since the analyses were unadjusted and therefore prone to confounding. Furthermore, among the five studies in the general adult population, three were conducted in pregnant women and one in non-pregnant pre-menopausal women, thus restricting the scope of generalizability of the results. Only one study was conducted in non-pregnant adults that included men. The studies in non-occupationally (environmentally) exposed adults reported no statistically significant findings suggesting no relationship between body Pb burden and oxidative stress. But it is unclear whether the physiological status (e.g. pregnancy) of the participants masked any potential influence of Pb on the formation of oxidative stress markers or the body's ability to mount an antioxidant response. More studies with environmentally-exposed

adults (both during and outside of pregnancy) are clearly needed to better understand the relationship between body Pb burden and oxidative stress.

4.3. Measurement issues with biomarkers of oxidative stress-

The best method for the accurate assessment of oxidative stress in biological samples is an active area of research (Halliwell, 1993, 2000; Jones, 2006). In addition to accuracy of the method of assessment, specificity, sensitivity and clinical relevance of a biomarker are of equal importance (Dalle-Donne et al., 2006). Direct assessment of oxidative stress includes the measurement of free radicals by spin-trapping and electron-spin resonance spectroscopy, but this method is not suitable for epidemiological studies due to extremely unstable nature of the free radicals and the complexity of the assays (Jones, 2006). Therefore, the focus of the measurement has been the assessment of damage to macromolecules (lipid, protein, DNA) or the concentration of antioxidants, and the activities of antioxidant enzymes. Measures of MDA and isoprostane in plasma, serum or urine are considered reliable indicators of lipid peroxidation *in vivo* (Halliwell & Chirico, 1993; Morrow, 2005), with more importance now being given to isoprostanes (Basu, 2004; Morrow, 2005). Although a few studies in adults measured isoprostanes, all the studies in children that examined lipid peroxidation assessed concentrations of MDA. Moreover, the thiobarbituric acid (TBA) test is performed to measure the amount of MDA in a sample (mostly plasma). In the test, TBA reacts with MDA molecules and produces a colored complex that can be measured by absorption spectrometry. Although the method is rapid and relatively simple, it is non-specific, since TBA can react with many other molecules such as bilirubin, ribose, amino acids, pyrimidines and sialic acid (Halliwell & Chirico, 1993). Several methods using high-performance liquid chromatography (HPLC) to measure MDA have been developed to overcome this problem (Halliwell & Chirico, 1993). Furthermore, the measurement of both MDA and

isoprostanes in urine may minimize the artifactual production of these markers during sample collection/processing/storage (Martin & Boyd, 2010). The 8-OH-dG as a biomarker for oxidative stress and specifically for DNA oxidation has gained a great deal of attention (Cooke et al., 2008; Garatt et al., 2010) and is now being used in studies with children (Svecova et al., 2009). However, among the studies reviewed here 8-OH-dG was only used in adults. Although several methodological considerations may arise while choosing the analytical technique for 8-OH-dG in biological specimens, this biomarker can be very useful because it is related to many disease outcomes (Dalle-Donne et al., 2006; Garatt et al., 2010).

4.4. Future Research directions:

Additional studies in children and adults with chronic low-level environmental Pb exposure are necessary to understand the relation between body Pb burden and oxidative stress. Well-designed studies including prospective cohorts with multiple measurements at different time points will be better suited to detect the response of the antioxidant system to Pb exposure and characterize these relationships in terms of timing and severity. Because most of the pediatric population is now experiencing low-level exposure to Pb, it is particularly important to focus the future investigations on this group. If low-level exposure is indeed associated with oxidative stress, which could in term be shown to mediate the association between lead exposure and chronic disease states, the public health implications of this research are enormous.

While careful consideration should be given to the choice of biomarker of oxidative stress, appropriate statistical approaches are important to test the hypothesis that Pb exposure contributes to oxidative stress. For example, adequate information on the factors that could be independently related to body Pb burden and oxidative stress (such as socio-economic status, exposure to other toxicants, smoking, age, sex, body mass index, physical activity, individual

genetic variations and diet) should be collected and taken into account in statistical analyses. In addition, appropriate statistical modelling is needed to understand how effect modification/interactions between Pb and other factors (dietary, genetic and environmental) influence the direction and strength of the association between Pb and oxidative stress. Additional thoughts should be given to building mediation models. For example the antioxidants or antioxidant enzymes could mediate the association between body Pb burden and free-radical mediated oxidation products of lipids, proteins or DNA.

Finally, studies designed to understand the functional consequences of Pb-induced oxidative stress are required to determine the relevance of this mechanism to Pb toxicity in humans. For example, research is needed to understand whether Pb-induced oxidative stress is in the pathway between body Pb burden and disease outcome such as neuro-behavioral deficits or cardiovascular problems.

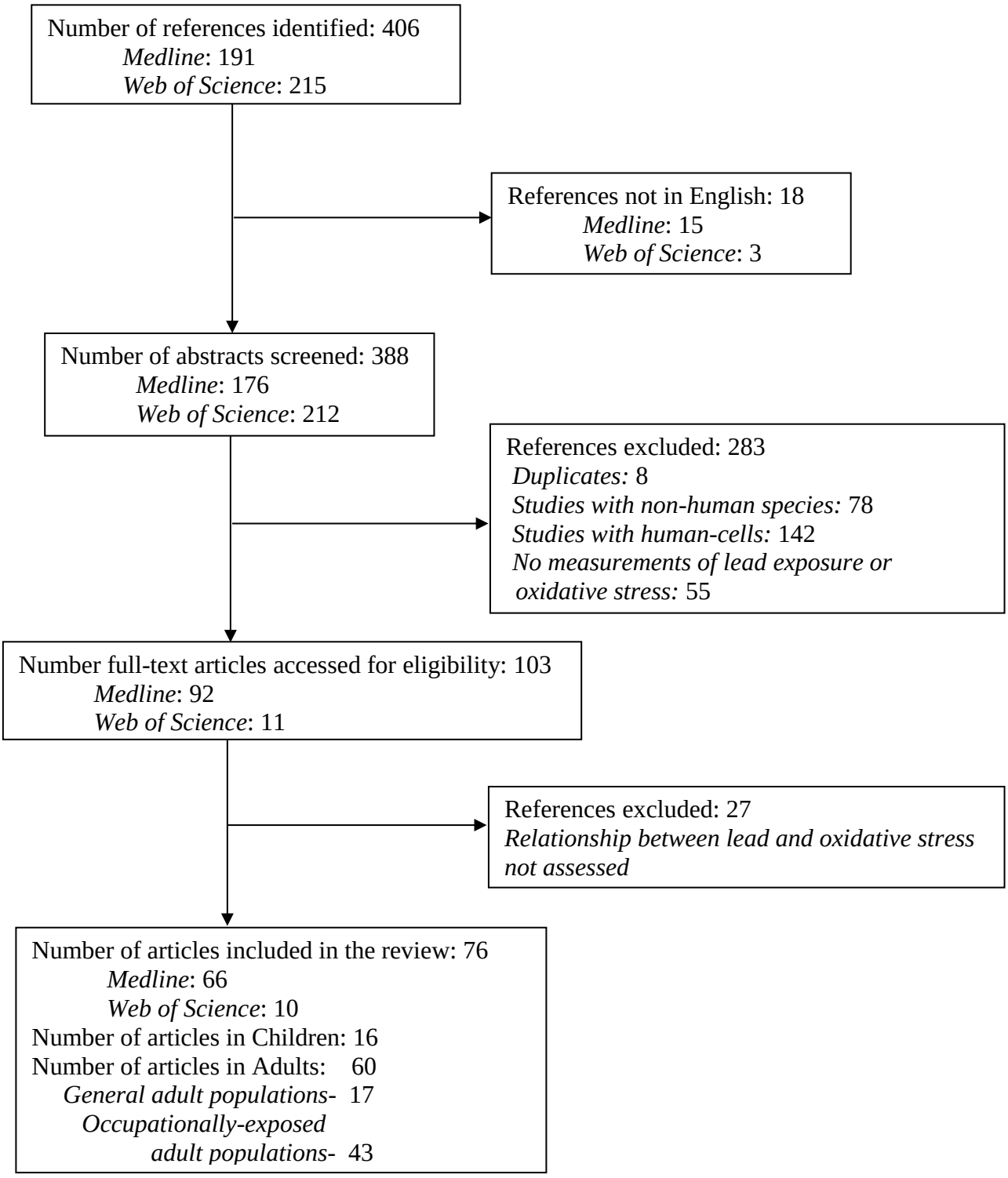

Figure 1: Flow-diagram of study selection process

Table 3: Studies on lead exposure and oxidative stress in children

Author/s	Year	Study-type	Country	Study population	Lead (Pb) assessment	Oxidative Stress (OS) measures	Main findings	Statistical analysis adjusted for?
Osman et al	1998	Cross-sectional	Sweden	4-14 y olds living in three towns of Katowice industrial region; n= 157	Blood lead level (BLL)- Median [range]: 7.3 [1.9 – 29.2] µg/dL‡	Blood and serum selenium (Se) levels, GPx activity	Negative correlation between BLL, Se levels in blood and serum, and GPx activity	No
Yanez et al	2003	Cross-sectional	Mexico	3-6 y olds attending kindergartens in two towns- one near a mining site and the other 15 km off the mining site; n= 55	BLL- Geometric mean [range]: Children near the mine: 11.6 [3.0-19.5] µg/dL Children away from the mine: 8.3 [3.0 – 25.0] µg/dL	DNA damage (Comet assay)	Higher DNA damage in children living near the mining site than those living away from the mining site. No association between BLL and DNA damage was assessed	No
Oktem et al	2004	Cross-sectional	Turkey	15-19 y old auto-repair workers (n= 79), and age, sex and demographically matched healthy control subjects (n= 71)	BLL- Mean ± SD: Workers- 7.8 ± 3.8 µg/dL Non-occupationally-exposed controls- 1.6 ± 0.8 µg/dL	Erythrocyte MDA levels, activities of SOD, CAT and GPx	Elevated MDA levels and GPx activity in workers compared to control. Positive correlations between BLL and MDA or GPx	No
Ahamed et al	2005	Cross-sectional	India	Children (4-12 y) from rural and urban areas around Lucknow city; n= 62	BLL- Mean ± SD: 7.5 ± 3.1 µg/dL	MDA, GSH levels and CAT activity	BLL was positively correlated with MDA and CAT activities; and inversely related to GSH activity	No
Ahamed et al	2006	Cross-sectional	India	Adolescent boys (15-18 y old) living in Lucknow city; n= 39	BLL- Mean ± SD: 9.96 ± 3.63 µg/dL	MDA, GSH levels and CAT activity	When OS measures were compared between BLL< 10 and >10 µg/dL (previous CDC level of concern), MDA levels and CAT activity were significantly higher among children with BLL> 10 µg/dL than children with BLL< 10 µg/dL. No difference in GSH levels between the groups. Positive associations between BLL and MDA, and BLL and CAT activity in unadjusted regression analyses	No

Table 3 continues…

Author/s	Year	Study-type	Country	Study population	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Diouf et al	2006	Cross-sectional	Senegal	8-12 y olds living in a rural and an urban area of Senegal; n= 330	BLL- Mean ± SD: Rural area: 5.2 ± 5.9 µg/dL Urban area: 9.9 ± 3.9 µg/dL	GPx, SOD, GR activities, serum Se levels and glutathione status (GSSG/GSH)	BLL negatively correlated with GPx activity and Se levels. No significant associations of BLL with other parameters of OS	No
Jin et al	2006	Cross-sectional	China	Pre-school children (3-6 y) from a city with steel-refinery; n= 408	BLL- Geometric mean ± SD: 6.98 ± 1.75 µg/dL	Plasma MDA, GSH levels, and activities of SOD, GPx	Significantly higher levels of MDA in children with BLL≥ 10 µg/dL than those with BLL< 10 µg/dL. No significant differences in activities of SOD and GPx or GSH levels among children with different BLLs	No
Mielzynska et al	2006	Cross-sectional	Poland	5-14 y old children from two cities with known history of Pb exposure and air pollution; n= 74	BLL- Mean ± SD: 7.7 ± 4.3 µg/dL	DNA damage measured by MN SCE levels in peripheral lymphocytes	Positive association between BLL and number of MN per 1000 cells; no association between BLL and SCE	Yes age, sex, parental education, exposure to environmental tobacco smoke and indoor emission from coal-burning stoves
Yetkin-Ay et al	2007	Cross-sectional	Turkey	Male 16 y old apprentices (n= 30) working in auto-repair workshop, and age, sex and SES matched control subjects (n= 30)	BLL- Mean ± SD: Workers: 7.38 ± 4.41 µg/dL Controls: 2.27 ± 1.49 µg/dL	Erythrocyte MDA level, activities of SOD, CAT and GPx	No significant differences in OS measures between the two groups; BLL and OS measures not correlated	No
Ahamed et al	2008	Case-control	India	Cases: 3-12 y olds with neurological disorders (cerebral palsy, seizures and encephalopathy); n=30 Controls: age and SES matched children living in and around Lucknow city (n= 60)	BLL- Mean ± SD: Cases- 7.7 ± 2.3 µg/dL; Non-occupationally-exposed controls- 6.9 ± 2.9 µg/dL	Blood MDA, GSH levels, SOD, CAT and glutathione peroxidase (GPx) activities	A significant positive relation between BLL and MDA levels, BLL and activities of SOD and CAT, but not GPx. An inverse relation between BLL and GSH	No

Table 3 continues…

Author/s	Year	Study-type	Country	Study population	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Ergurhan-Ilhan et al	2008	Cross-sectional	Turkey	Male 16 y old apprentices (n= 25) from an auto-repair factory and age, sex and SES matched control subjects from the city of Isparta (n= 24)	BLL- Mean ± SD: Workers- 7.9 ± 5.2 µg/dL Non-occupationally-exposed controls- 2.6 ± 2.0 µg/dL	Activities of SOD, GPx and CAT, levels of α-tocopherol, β-carotene, and MDA in erythrocyte	Higher GPx activity, MDA levels and lower levels of α-tocopherol and β-carotene in the apprentices than in the controls; no significant differences in SOD or CAT activities among the groups; no relation between BLL and OS measures	No
Mendez-Gomez et al	2008	Cross-sectional	Mexico	Children (6-11 y) from 3 schools located near, intermediate and distant sites from a smelter complex; n= 65	BLL- Median (Range): Children near the smelter: 28.6 (11.4-47.5) µg/dL, intermediate: 19.5 (11.3-49.2) µg/dL and distant site from the smelter: 4.6 (0.1-8.7) µg/dL	DNA damage (tail length) and DNA repair in lymphocytes	DNA damage was significantly higher in children who attended the school nearest to the smelter than those who went to more distant schools. No significant association between BLL and DNA damage or repair	Yes age, sex, self-reported use of Pb glazed pottery by families and children's urinary arsenic concentration
Ahamed et al	2011	Case-control	India	Cases: 3-12 y old children with aplastic anemia (n= 17) Controls: age-sex matched healthy children living in and around Lucknow city (n= 51)	BLL- Mean ± SD: Cases- 9.86 ± 2.04 µg/dL; Controls: 4.23 ± 1.23 µg/dL;	TBARS, GSH levels in blood and CAT activity	TBARS levels and CAT activity were significantly higher whereas GSH levels were lower in cases than the controls. Positive correlation between BLL and TBARS, and BLL and CAT activity	No
Cabral et al	2012	Cross-sectional	Senegal	Children (1-16 y) from two communities- one near a landfill site (n= 26) and the other 3.5 km away from the landfill (n= 32)	BLL- Mean ± SD Children living near the landfill: 14.97 ± 3.16 µg/dL Children living away from the landfill: 8.22 ± 9.7 µg/dL	Plasma MDA and glutathione status (GSSG/GSH)	Children living near the landfill had significantly higher MDA levels than those living 3.5 km off the landfill. No difference in glutathione status between the groups. BLL positively correlated with MDA	No

Table 3 continues…

Author/s	Year	Study-type	Country	Study population	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Jasso-Pineda et al	2012	Cross-sectional	Mexico	4-11 y olds living in 3 areas- one at a mining site, the other two at sites 8 km and 200 km away from the mining site	BLL- Geometric Mean ± SD: Mining site: 11.4 ± 0.6 µg/dL 8km away from mine: 7.3 ± 1.5 µg/dL 200 km away from mine: 5.3 ± 0.5 µg/dL	Comet assay for measuring DNA damage and DNA repair	Higher DNA damage and lower DNA repair in children living at the mining site (higher mean BLL) than those living 200 km off the mine (lower mean BLL). Association between BLL and DNA damage did not reach statistical significance	Yes age, sex, height and nutritional status
Martínez et al	2013	Cross-sectional	Argentina	1 month – 14 y old children from the city of Cordoba, (n= 161)	Geometric mean ± SD: 2.6 ± 0.3 µg/dL	Activities of SOD and CAT	No significant relation between BLL and CAT or SOD activities	No

Abbreviations:
BLL- whole blood lead level
Lipid peroxidation markers: MDA-malonaldehyde, TBARS- thiobarbituric acid reactive substances
DNA damage: MN- micronuclei, SCE- sister chromatid exchange
Anti-oxidant enzymes: SOD- superoxide dismutase, CAT- catalase, GPx- glutathione peroxidase, GR- glutathione reductase
Non-enzymatic antioxidant: GSH- glutathione, GSH/GSSG- ratio of reduced/oxidized glutathione, α-tocopherol, β-carotene, Se- selenium
[ɨ]values converted from µmol/L to µg/dL (0.48 µmol/L= 10 µg/dL)

Table 4: Studies on lead and oxidative stress in adults

Author/s	Year	Study-type	Country	Study population	Lead (Pb) assessment	Oxidative stress (OS) assessment	Main findings	Statistical analysis adjusted for?
Non-occupationally-exposed general populations								
Lin et al	1996	Case-control	Taiwan	Cases: Patients (28-79 y) with chronic renal failure (n=26) Controls: Age-sex-matched healthy adults (n=25)	Erythrocyte Pb [Mean ± SD]: Cases: 175 ± 54 µg/L (before dialysis) 188 ± 61 µg/L (after dialysis) Controls: 90 ± 20 µg/L	Plasma MDA and selenium (Se) levels	Higher MDA and lower Se levels in cases than controls. Positive correlation between erythrocyte Pb and plasma MDA levels	No
Merzenich et al	2001	Cross-sectional	Germany	35-80 y old residents of the city of Bremen (n= 227)	BLL [Median (range)]: 4.6 (2.0-15.6) µg/dL	8-oxo-Gua levels, oxidative DNA lesions in lymphocytes	No significant association between BLL and lymphocytic DNA damage measures	Yes Urinary levels of Ni, Cr, Cd, age, sex, occupational exposure to X-ray, external seasonal influences
Xu et al	2003	Cross-sectional	China	26-45 y old non-smoking, healthy men	Seminal plasma Pb level [Geometric mean (95% confidence interval)]: 7.8 (4.6-13.1] µg/L	8-OHdG levels in seminal plasma	Positive correlation between Pb and 8-OHdG levels in seminal plasma	No
Jurasovic et al	2004	Cross-sectional	Croatia	19-48 y old men attending an andrology unit in a clinic; n=123	BLL [Median (range)]: 5.7 (2.5-14.9) µg/dL	Blood GPx activity, serum Se level	No significant correlation between BLL and GPx activity or serum Se levels	No
Lee et al	2006	Cross-sectional	US	>20 y old non-pregnant adults participating in NHANES III; n=10,098	BLL [Geometric mean (range)]: 2.8 µg/dL (0.7-56 µg/dL)	Serum GGT, Vit C, carotenoids, Vit E	BLL positively associated with serum GGT and inversely related to vit C, E and carotenoids.	Yes age, race, sex, SES
Anis et al	2007	Case-control	Egypt	Cases: Men (34-70y) with erectile dysfunction; n= 34 Controls: Age-matched healthy men; n= 15	BLL [Mean ± SD]: Cases: 34.8 ± 38.2 µg/dL Controls: 3.1 ± 3.2 µg/dL	Serum MDA, hydrogen peroxide, SOD, CAT, GPx, Vit C	Men with BLL≥ 25 µg/dL had higher MDA, hydrogen peroxide and lower SOD, CAT and GPx than those with BLL< 25 µg/dL. No significant difference in serum Vit C between two groups.	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Study-type	Country	Study population	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Non-occupationally exposed general populations								
Kiziler et al	2007	Case-control	Turkey	Cases: Men (mean age 40 y) suffering from infertility; n= 50 Controls: Age-matched men with no infertility problems; n= 45	BLL [Mean ± SD]: Cases: 36.8 ± 12.3 µg/dL Controls: 23.2 ± 5.6 µg/dL Seminal plasma Pb [Mean ± SD]: Cases: 38.2 ± 11.3 µg/dL Controls: 26.3 ± 5.2 µg/dL	Spermatozoa ROS, seminal plasma MDA, protein carbonyl, GSH, GST activity	Higher ROS, MDA and protein carbonyl and lower GSH, GST activity in cases than controls. Positive correlation between seminal plasma Pb and spermatozoa ROS	No
Pizent et al	2008	Cross-sectional	Croatia	19-67 y old office workers with no history of occupational exposure; n= 216 (men= 50, women= 166)	BLL [Median (range)]: Men: 3.17 (0.99 – 7.23) µg/dL Women: 2.16 (0.56–7.35) µg/dL	Serum Se levels, activities of SOD and GPx	Negative correlation between BLL and SOD activity in both men and women	No
Ahamed et al	2009	Case-control	India	Cases: women with preterm babies Controls: age-matched women with full-term deliveries	Placental Pb (Mean ± SD): Study subjects- 0.39 (2.7) µg/g wet tissue Controls- 0.27 (0.1) µg/g wet tissue	TBARS, GSH, activities of SOD, CAT, GPx, GR, GST	Higher TBARS and activities of SOD, CAT, GPx and GR while lower GSH in the placenta of women with the preterm deliveries as compared to the full-term deliveries. Positive correlation of placental Pb with TBARS, SOD and CAT, and inverse correlation with GSH.	No
Wu et al	2009	Cross-sectional	Taiwan	Immigrant women (mean age 27.1 y) settled in Taiwan (n=71) and native women of same age range living in the same area (n= 83)	BLL (Mean ± SD): Immigrants: 2.23 ± 1.63 µg/dL Natives: 1.63 ± 1.00 µg/dL	DNA damage (comet assay)	Positive association between BLL and DNA damage (Tail DNA% and tail moment)	Yes age, family income, work history in Pb factories (yes or no), country of origin, number of pregnancies, medication in past three days (yes or no), supplement use (yes or no)
Engstrom et al	2010	Cross-sectional	Bangladesh	Pregnant women (19-35 y) exposed to high levels of arsenic; n= 172	BLL [Median (range)]: 79 (48-150) µg/Kg	Urinary 8-OHdG	No significant association between BLL and 8-OHdG.	Yes age, gestational week, blood levels of zinc, selenium, manganese and plasma ferritin

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Study-type	Country	Study population	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Non-occupationally exposed general populations								
Komatsu et al	2011	Cross-sectional	Mongolia/ Japan	10-82 y old (mean age 39.7 y) Mongolians (n= 244) including healthy and non-healthy subjects (Parkinsonism and arthritis) and healthy Japanese subjects (mean age 45.7 y) (n= 81)	Hair Pb [Mean ± SD]: Healthy Mongolian subjects: 1789 ± 1705 ppb Healthy Japanese subjects: 548 ± 438 ppb	Urinary 8-OHdG	Higher urinary 8-OHdg in Mongolian healthy subjects than Japanese healthy controls. Positive correlation between hair Pb and urinary 8-OHdG	No
Pollack et al	2012	Longitudinal	US	Healthy premenopausal non-pregnant women (18-44 y); n= 252	BLL [Median (IQR)]: 0.86 (0.67-1.20) µg/dL	Plasma F_2-8α isoprostanes (isoprostane), 9-HODE, 13-HODE, and TBARS levels	No association between BLL and plasma levels of isoprostane, 9-HODE, 13-HODE and TBARS	Yes age, BMI, smoking status, race
Sirfim et al	2012	Cross-sectional	Portugal	Healthy pregnant women (17-40 y) n= 109	Placental Pb level (Mean ± SD): 0.039 ± 0.006 µg/g wet weight (w.w.)	Placental lipid peroxidation (LPO)- sum of MDA and 4-hydroxyalkenals (4-HNE) per gram of total protein	In a cluster analysis, women in a cluster with highest placental Pb (average: 0.051 µg/g w.w.) had higher LPO compared to the cluster with lowest placental Pb (average: 0.029 µg/g w.w.); Pb levels in placenta positively correlated with LPO	No
Bakheet et al	2013	Cross-sectional	Saudi Arabia	Healthy men (20-43 y) from two cities- one with a gold mine (n= 40) and the other without a mine; n= 20	BLL (Mean ± SD): Men living in a city with a gold mine: 2.1 ± 0.2 µg/dL Men from a city without a mine: 1.1 ± 0.06 µg/dL	Plasma 8-OHdG	No significant difference in 8-OHdG between Pb-exposed group (Mahd Ad-Dahab city) and the controls (Riadh city)	No
Mancinelli et al	2013	Cross-sectional	Italy	Alcoholic patients (mean age 46.5 y) admitted to a day hospital for treatment and rehabilitation (n= 58)	BLL [Mean ± SD]: 6.98 ± 4.10 µg/dL	Serum ROM	Positive correlation between BLL and serum ROM level	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Study-type	Country	Study population	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Non-occupationally exposed general populations								
Pandya et al	2013	Cross-sectional	India	Benign Prostate Hyperplasia patients (55-85 y); n= 116	Pb level in prostate tissue samples (descriptive statistics on Pb values not reported)	GSH, LPO levels and activities of SOD, CAT & GPx in mitochondrial & post-mitochondrial fraction of prostate tissues	Significant negative correlation between Pb and GPx activity in prostate tissues	No

Occupationally-exposed populations								
Author/s	**Year**	**Country**	**Study population**	**Non-occupationally-exposed controls**	**Lead (Pb) assessment**	**Oxidative stress (OS) measures**	**Main findings**	**Statistical analysis adjusted for?**
Jiun et al	1994	Taiwan	Male workers (22-64 y) with no history of chronic diseases, working in an industrial area; no additional information on types of job or the industry where subjects worked; n= 62	19-69 y old men (n= 26) and women (n= 40); no information available on selection of controls	BLL (Mean ± SD): Workers- 37.2 ± 12.5 µg/dL Controls- 13.4 ± 7.5 µg/dL	Plasma MDA levels	Higher plasma MDA in those with BLL> 35 µg/dL compared to those with BLL≤ 35 µg/dL; positive correlation between BLL and plasma MDA in unadjusted regression analysis	No
Chiba et al	1996	Japan	Male steel rope factory workers (age range unknown) working with lead furnace; n= 63	Male workers not working with lead furnace; no additional information available on control subjects; n= 7	BLL (Mean ± SD): Workers- 17.8 ± 11.5 µg/dL Controls- 5.7 ± 4.3 µg/dL	Activities of total SOD, Mn-SOD, Cu,Zn-SOD, CAT and GPx in plasma and erythrocytes	Compared to BLL< 10 µg/dL, those with BLL> 20 µg/dL had higher CAT activity. No difference in CAT activity between BLL< 10 µg/dL and BLL≤ 20 µg/dL. No correlation between BLL and antioxidant enzyme activities	No
Costa et al	1997	Brazil	Men (18-53 y) from pottery-manufacturing plant (n= 60)	age-matched healthy males (n= 30)	BLL (Mean ± SD): Workers- 53.4 ± 1.21 µg/dL Controls- 6.3 ± 2.3 µg/dL	SOD activity, urinary chemiluminiscence (CL)	Significant positive correlations between BLL and urinary CL and SOD activity	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Ye et al	1999	China	Lead-smelter workers (n= 66) (age not reported)	healthy adults (n= 28)	BLL (Range): ~13.0 to ~37.0 µg/dL	Plasma MDA, SOD activity, DNA damage (comet assay)	Positive correlation of BLL with MDA, SOD activity and DNA damage. Compared to lower BLL (~13 µg/dL), higher grades of DNA damage with higher BLL (27-37 µg/dL).	No
Dursun et al	2001	Turkey	31-47 y old, male metal powder factory workers; n= 31	30-49 y old, male sugar-refining factory workers; n=19	BLL (Mean ± SD): Workers: 15.0 ± 10.1 µg/dL Controls: 2.4 ± 0.9 µg/dL	Plasma and erythrocyte LPO levels	Plasma and erythrocyte LPO were higher in workers than in controls. Positive correlations between BLL and plasma and erythrocyte LPO in workers and controls	No
Vaglenov et al	2001	Bulgaria	Male workers (mean age 39.1 y) employed in a storage battery plant; n= 103	Male administrative and maintenance stuffs (mean age 42 y) from the same or different plant; n= 78	BLL (Mean ± SD): Workers: 56.25 ± 2.08 µg/dL[ǂ] Controls: 18.96 ± 0.83 µg/dL[ǂ]	MN frequency in binucleated cells	Higher MN frequency and number of binucleated cells presenting one or more micronuclei (BNMN) in workers vs controls groups. Positive correlation between BLL and BNMN	No
Danadevi et al	2003	India	19-45 y old male workers working in a secondary Pb recovery unit involved in the extraction of Pb from batteries; n= 45	Age and SES matched men involved in administrative jobs in offices surrounding the factory; n= 36	BLL (Mean ± SD): Workers: 24.83 ± 14.66 µg/dL Controls: 2.75 ± 1.52 µg/dL	DNA damage (comet assay)	Higher DNA damage in workers than in controls. BLL positively correlated with DNA damage	No
Hengstler et al	2003	Germany	Workers (age not reported) employed in factories involved in production of batteries, galvanization, and recycling of electronic goods; n= 78	Age matched healthy adults who were medical students, working in offices and laboratories; n= 22 (control group not used for assessing relationships between Pb and DNA damage)	BLL [Median (range)]: Workers: 4.41 (2.84 – 13.68) µg/dL	DNA damage (DNA single strand break analysis)	BLL was not associated with DNA damage	Yes Age, gender, cigarette and alcohol use, serum iron levels, cadmium levels in air and blood, and urinary cobalt levels

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Palus et al	2003	Poland	26-52 y old male workers working in battery plants; n= 44	21-56 y old men and women engaged in office works in the plant; n= 52	BLL (Mean ± SD): Workers: 50.4 ± 9.2 µg/dL Controls: 5.6 ± 2.8 µg/dL	DNA damage (MN frequency, SCEs, and comet assay)	Higher MN frequency and SCEs in workers than in controls	No
Garcon et al	2004	France	Workers (33-55y) from a non-ferrous metal smelter (n=35)	No control group	BLL[Mean (range)]: 39.57 (10.0-61.0) µg/dL UPb [Mean (range)]: 95.2 (4.7-1286.1) µg/g creatinine	Urinary 8-OH-dG, plasma MDA, Se concentrations, activities of SOD, GPx, GR and GSSG/GSH ratio	Positive association of BLL with MDA and GPx activity. No statistically significant correlation between UPb and 8-OH-dG; relation between BLL and 8-OH-dG not reported	Yes Smoking behavior
Gurer-Orhan et al	2004	Turkey	Male (mean age 32 y) battery plant workers; n= 20	age-sex-matched controls; n= 16	BLL (Mean ± SD): Workers: 54.6 ± 17 µg/dL Controls: 11.8 ± 3.2 µg/dL	Plasma MDA, activities of CAT and G6PD, GSH/GSSG ratio	Significantly higher MDA, CAT and G6PD activities and lower GSH/GSSG ratio in workers than controls; statistically significant positive correlation of BLL with MDA, CAT, G6PD and GSH/GSSG ratio	No
Kasperczyk et al	2004	Poland	40 y old workers employed in zinc and Pb steel works; n= 137	Age matched administrative staffs; n= 35	BLL (Mean ± SD): Low-Pb exposed workers: 35.0 ± 9.3 µg/dL High-Pb exposed workers: 44.2 ± 8.1 µg/dL Controls: 8.6 ± 2.4 µg/dL	MDA levels in erythrocytes, activities of GR, GPx	Higher erythrocyte MDA levels in high-Pb exposed workers than low-Pb exposed workers and controls. Higher GPx activity in low-Pb exposed workers compared to controls, lower GPx activity in high-exposed workers compared to controls	No
Kasperczyk et al	2004	Poland	40 y old workers employed in zinc and Pb steel works; n= 137	Age matched administrative staffs; n= 35	BLL (Mean ± SD): Workers: 39.1 ± 7.5 µg/dL Low Pb-exposed group- 30.3 ± 2.9 µg/dL High Pb-exposed group- 43.1 ± 5.2 µg/dL Controls: 9.2 ± 2.7 µg/dL	Serum levels of MDA, total activity of SOD, activity of Mn-SOD and CAT	Higher serum MDA in workers than in controls. Positive correlation between BLL and SOD activity	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Li et al	2004	China	Welders from automobile factory; n= 37	Workers from a food factory; n= 62	BLL (Mean ± SD): Welders- 3.1 ± 0.2 µg/dL Controls- 1.2 ± 0.8 µg/dL	Serum MDA and activity of SOD in erythrocytes	Higher levels of MDA and lower SOD activity in welders compared to controls	No
Han et al	2005	South-Korea	34-37 y old male welders working in a shipyard; n= 197	Age matched office workers from the same industrial complex; n= 150	BLL [Geometric mean (95% confidence interval)]: Welders: 0.53 (0.51-0.54) µg/dL Controls: not reported	ROS, serum isoprostanes, total antioxidant status (TAS), activities of GPx, and SOD	Higher serum isoprostane levels, TAS and GPx activity in welders than controls	No
Karakaya et al	2005	Turkey	32-47 y old, male, battery manufacturing workers; n= 23	Age matched male office employees; n= 23	BLL (Mean ± SD): Workers: 72.7 ± 34.1 µg/dL Controls: 4.4 ± 2.1 µg/dL	DNA damage (Chromosomal aberrations)	Higher chromosomal aberrations in workers than in controls	No
Kasperczyk et al	2005	Poland	Normotensive and hypertensive male workers (mean age 40 y) from a zinc and lead steelworks; n= 185	Age matched administrative staffs; n= 35	BLL (Mean ± SD): Low-Pb exposed workers: 33.4 ± 4.3 µg/dL High- Pb exposed workers: 46.2 ± 3.78 µg/dL Controls: 9.2 ± 2.7 µg/dL	Plasma and erythrocyte TBARS levels	Higher TBARS in workers than controls. Positive correlation between BLL and TBARS in erythrocytes	No
Patil et al	2006	India	20-40 y old male silver jewelry workers; n=30	age matched men; n= 35	BLL (Mean ± SD): Workers: 48.6 ± 7.4 µg/dL Controls: 12.5 ± 4.1 µg/dL	Levels of MDA and ceruloplasmin; and activities of SOD and CAT	Significantly elevated MDA, ceruloplasmin levels and lower antioxidant enzyme activities in workers than in controls	No
Patil et al	2006	India	20-40 y old male battery manufacturing workers; n=28	Age matched healthy men; n= 35	BLL (Mean ± SD): Workers: 53.6 ± 16.9 µg/dL Controls: 12.5 ± 4.1 µg/dL	Serum MDA levels, activities of SOD and CAT	Higher MDA levels and lower activities of SOD and CAT in workers than in controls. Positive correlation between BLL and MDA in workers but not in controls	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Garcon et al	2007	France	31-57 y old male smelter-workers; n= 57	age matched male controls; n= 57	BLL (Mean ± SD): Workers- 38.71 ± 9.91 µg/dL Controls- 5.64 ± 3.51 µg/dL	Levels of plasma MDA and selenium (Se), urinary 8-OH-dG levels, and activities of SOD, GPx and GR	MDA was significantly higher and Se levels was lower in workers than in controls; no difference in enzyme activities or urinary 8-OH-dG levels among groups; positive correlation of BLL with MDA and SOD, and inverse relation with Se; relation between BLL and 8-OH-dG not reported	No
Devi et al	2007	India	Workers (mean age 40 y) engaged in road-work and exposed to fumes from tail pipes of engines; n= 100	Age, SES and demographically matched adults; n= 50	BLL (descriptive data on BLL not provided)	Serum MDA and GSH levels, GST and CAT activity	No correlation between BLL and OS parameters	No
Hsu et al	2007	Taiwan	Male battery plant workers (mean age 29 y); n= 80	No control group	BLL (Mean ± SD): 40.2 ± 12.8 µg/dL	Sperm chromatin DNA structure	Positive correlation between BLL and sperm chromatin DNA denaturation	Yes Smoking status
Quintanar-Escorza et al	2007	Mexico	Male workers (mean age 27 y) from an automobile battery recycling factory; n=15	Age, sex and SES matched healthy men; n =15	BLL (Mean ± SD): Workers: 74.4 ± 21.9 µg/dL Controls: 9.9 ± 2.2 µg/dL	Erythrocyte TBARS, protein carbonyl levels	Higher TBARS and protein carbonyl contents in workers than in controls. Positive correlation between BLL and TBARS in workers and controls	No
Kasperczyk et al	2008	Poland	Healthy, non-smoking, male workers (mean age 36 y) from zinc and lead steel works; n= 63	Office workers (mean age 34 y); n=14	BLL (Mean ± SD): Moderate-Pb exposed workers: 34.7 ± 0.8 µg/dL High-Pb exposed workers: 53.1 ± 2.1 µg/dL Controls: 8.5 ± 0.5 µg/dL	MDA levels in seminal plasma	High Pb exposed group had higher sperm MDA levels than controls. Positive correlation between BLL and MDA levels in seminal plasma	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Khan et al	2008	Pakistan	Industrial workers engaged in smelting operation; n= 87	age and sex matched office staffs from the factory; n= 61	BLL [Median(range)]: Workers- 29.1 (9.0 - 61.1) µg/dL Controls- 8.3 (1.0 – 21.7) µg/dL	Serum MDA levels and gamma glutamyl transferase (GGT)	Higher serum MDA and GGT levels in workers than the controls; significant positive correlation between BLL and MDA and GGT	No
Mohammad et al	2008	India	20-50 y old residential or commercial painters; n= 35	age and sex-matched subjects; n= 35	BLL (Mean ± SD): Cases- 21.92 ± 6.19 µg/dL Controls- 3.06 ± 1.01 µg/dL	TBARS levels and activities of GSH, GSSG, SOD and CAT	Activities of SOD, CAT and GSSG were significantly lower whereas GSH and TBARS levels were higher in painters than in controls; relation between BLL and OS measures not examined	No
Kasperczyk et al	2009	Poland	Normotensive and hypertensive workers (mean age 41 y) employed in a metal workshop; n= 92	Age matched office staffs; n= 30	BLL (Mean ± SD): Normotensive workers: 41.8 ± 5.0 µg/dL Hypertensive-I workers: 45.3 ± 4.7 µg/dL Hypertensive-II workers: 42.7 ± 8.1 µg/dL Controls: 7.7 ± 1.7 µg/dL	Erythrocyte MDA levels, activities of SOD, CAT and GPx	Higher MDA levels and GPx and SOD activities in normotensive workers than in controls. Higher MDA and lower activities of GPx in hypertensive workers than controls	No
Grover et al	2010	India	Factory workers (mean age 31 y) involved in Pb extraction and Pb-battery production; n= 90	age and sex matched adults; n= 90	BLL (Mean ± SD): Workers- 30.33 ± 2.09 µg/dL Controls- 3.21 ± 0.26 µg/dL	Comet assay, micronucleus test (MNT), chromosomal aberration (CA) assay for DNA damage, activities of SOD, GPx and CAT; serum MDA levels	Higher comet tail length, increased frequency of MN and increased chromosomal damage in workers vs control subjects; significantly lower SOD, GPx and CAT activities and higher MDA levels in workers compared to the controls; relation between BLL and serum MDA not reported	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Malekirad et al	2010	Iran	Male workers (mean age 39 y y) working in a zinc-Pb mine; n= 67	Healthy adults (mean age 41 y) living in the nearby village; n= 67	BLL (Mean ± SD): Workers: 9.65 ± 3.28 µg/dL Controls- 5.07 ± 3.61 µg/dL	The activities of SOD, CAT, GR, myeloperoxidase (MPO), plasma 8-OH-dG levels, LPO and TAC	SOD, MPO, TAC and GR were significantly higher in workers than in controls; 8-OH-dG was higher in workers than in the control groups	No
Minozzo et al	2010	Brazil	18-55 y old male workers from an automobile battery recycling facility; n= 53	Age and SES matched healthy men; n= 53	BLL (Mean ± SD): Workers: 59.43 ± 28.34 µg/dL Controls: 2.44 ± 1.15 µg/dL	MN counts and DNA damage (comet assay)	Higher MN counts and DNA damage in workers than in controls. No correlation between BLL and MN counts or DNA damage.	No
Moro et al	2010	Brazil	Male, industrial painters (mean age 28 y); n= 48	Age matched non-occupationally exposed men; n= 30	BLL (descriptive data not provided)	Plasma MDA levels, SOD activity	Positive correlation between BLL and plasma MDA levels	No
Olewińska et al	2010	Poland	Male workers (mean age 39 y) involved in metal works; n= 62	age- matched administrative workers; n= 26	BLL (Mean ± SD): Workers- 45.76 ± 8.61 µg/dL Controls- 5.41 ± 2.27 µg/dL	Comet assay to measure DNA damage as the percentage of DNA in the tail, tail length and tail moment (TM)	Percentage of DNA in the tail, comet tail length and TM were higher in occupationally exposed workers with high mean BLL than in non-occupationally exposed control (low mean BLL); positive correlation of BLL with tail DNA% and TM	No
Conterato et al	2011	Brazil	21-49 year old male painters and battery-manufacturing workers from a battery plant; n =83	Healthy, age-matched adult men; n= 36	BLL (Mean ± SD) : Painters- 5.4 ± 0.4 µg/dL Battery workers- 49.8 ± 4.0 µg/dL Controls- 1.5 ± 0.1 µg/dL	Plasma MDA, protein carbonyl content, erythrocyte GSH, vitamin C levels, activities of GST, GPx, SOD, CAT, TrxR	No correlation between BLL and MDA levels. Protein carbonyl content inversely correlated with BLL. Significant positive correlation of BLL with Glutathione-S-transferase, GPx and SOD activities. No relation between BLL and CAT or TrxR activities or GSH levels; correlation between BLL and vitamin C non-significant	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Permpongpaibooon et al	2011	Thailand	Earthenware factory workers (mean age 41 y); n= 60	Age-matched general population; n= 65	BLL (Mean ± SD): Workers: 31.4 ± 2.5 µg/dL Controls: 3.9 ± 0.2 µg/dL	Conjugated diene (CD), total peroxides (TP), plasma MDA levels, total antioxidant status (TAS) and paraoxonase 1 (PON1) activity. Oxidative stress index (OSI) calculated from a percent ratio of TP to the TAS level	CD, TP, MDA and OSI levels were significantly higher and TAS or PON1 activities were significantly lower in factory workers (even in those with a BLL of 10-20 µg/dL) than their matched controls (BLL< 10 µg/dL); positive correlation of BLL with CD, TP, MDA and OSI, and a negative correlation with TAS or PON1 activity	No
Feksa et al	2012	Brazil	Male workers (mean age 35 y) from manufacturing and automobile factories; n= 22	Men (mean age 24 y) and women (mean age 29 y) with no history of occupational exposure; n= 21	BLL (Mean ± SD): Workers: 61.9 ± 10.3 µg/dL Controls: 1.9 ± 0.4 µg/dL	Erythrocyte GSH levels	Lower erythrocytic GSH levels in workers than in controls. Positive association between BLL and GSH levels in an unadjusted regression analysis	No
Garcia-Leston et al	2012	Spain	Workers (mean age 47.8 y) from chemical and lead-acid-battery manufacturing plants; n= 148	Administrative and commercial workers (mean age 43.1 y); n= 107	BLL (Mean ± SD): Workers: 32.0 ± 1.1 µg/dL Controls: 3.6 ± 0.4 µg/dL	MN frequency and DNA damage (comet assay)	Higher DNA damage and MN frequency in workers than controls. Positive correlation between BLL and DNA damage and BLL and MN frequency	No
Kasperczyk et al	2012	Poland	24-58 y old, healthy male workers from zinc and lead smelters; n= 45	21-60 y old, male Administrative workers; n= 17	BLL (Mean ± SD): Low-Pb exposed workers: 42.9 ± 10.3 µg/dL High-Pb exposed workers: 48.5 ± 7.1 µg/dL Controls: 5.7 ± 1.9 µg/dL	Activities of SOD, CAT and GPx	Higher activities of GPx and SOD in workers than in controls	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Kasperczyk et al	2012	Poland	22-58 y old male workers from zinc and lead smelters; n= 192	Age matched healthy male administrative workers; n= 73	BLL (Mean ± SD): Low-Pb exposed workers: 37.8 ± 10.0 µg/dL Moderate-Pb exposed workers: 47.9 ± 6.7 µg/dL High-Pb exposed workers: 49.8 ± 5.9 µg/dL Controls: 6.4 ± 2.5 µg/dL	Serum Se levels	Lower serum Se levels in workers than in controls	No
Kasuba et al	2012	Croatia	18-57 y old workers (employed for more than 6 y) from a ceramic factory; n= 30	Age, sex and smoking habit matched clerks and newly-hired workers; n= 30	BLL (Mean ± SD): Workers: 22.0 ± 1.8 µg/dL Controls: 3.0 ± 0.2 µg/dL	DNA damage (comet assay), MN frequency	Higher MN frequency and DNA damage in workers than in controls	No
Lin et al	2012	Taiwan	Workers (33-45 y old) from a glass factory; n= 97	Administrative workers; n= 33	Urinary lead level (UPb) (Mean ± SD): 18.2 ± 49.6 µg/g	Urinary concentration of 8-hydroxy-2’ deoxyguanosine (8-OHdG)	UPb not associated with 8-OHdG after adjusting for age, smoking behavior, and alcohol consumption	No
Bizon et al	2013	Poland	Male workers (mean age 40 y) employed in a copper smelter; n= 300	Age matched non-occupationally exposed men; n= 100	BLL (Mean ± SD): Non-smoking workers: 27.7 ± 11.9 µg/dL <20 cigarettes using workers: 29.8 ± 9.1 µg/dL ≥20 cigarettes using workers: 30.2 ± 8.5 µg/dL Non-smoking controls: 3.2 ± 8.9 µg/dL <20 cigarettes using controls: 3.7 ± 1.5 µg/dL ≥20 cigarettes using controls: 5.3 ± 5.2 µg/dL	Plasma MDA levels, erythrocyte GSH levels, activities of SOD, GPx and GST	Higher plasma MDA in workers than controls. Positive correlation between BLL and plasma MDA levels	No

Table 4 (Studies on lead and oxidative stress in adults) continues…

Author/s	Year	Country	Study population	Non-occupationally-exposed controls	Lead (Pb) assessment	Oxidative stress (OS) measures	Main findings	Statistical analysis adjusted for?
Occupationally-exposed populations								
Kasperczyk et al	2013	Poland	Male workers (23-59 y) employed in a zinc and lead factory; n= 125	Age matched healthy male administrative workers; n= 32	BLL (Mean ± SD): Workers: 26.1 ± 4.5 µg/dL Controls: 7.9 ± 2.4 µg/dL	Erythrocyte MDA levels	Higher erythrocyte MDA levels in workers than in controls. Positive correlation between BLL and MDA level	No
Prokopowicz et al	2013	Poland	Healthy workers (19-62 y) worked for at least 2 y in a metallurgic plant; n= 340	Age-sex-matched administrative workers; n= 61	BLL (Mean ± SD): Factory workers- Q1: 14.7 ± 3.3 µg/dL Q2: 26.2 ± 3.1 µg/dL Q3: 35.4 ± 2.4 µg/dL Q4: 47.3 ± 6.2 µg/dL Administrative workers- 3.2 ± 1.6 µg/dL	Serum levels of α-tocopherol and γ-tocopherol	Factory workers had higher γ-tocopherol than administrative workers. Factory workers in Q1 BLL had higher γ-tocopherol than Q2, Q3 and Q4. Significant negative association between BLL and γ-tocopherol but not with α-tocopherol	No

Abbreviations:
BLL: whole blood lead levels, UPb: urinary lead levels
ROS: Reactive Oxygen Species; ROM: Reactive Oxygen Metabolites
Lipid peroxidation markers- MDA: malonaldehyde, TBARS: thiobarbituric acid reactive substances, 9-HODE: 9-hydroxy-10,12-octadecadienoic acid, 13-HODE: 13-hydroxy-9,11-octadecadienoic acid (13-HODE),
DNA oxidation markers- 8-oxo-Gua: 7,8-dihydro-8-oxoguanine; 8-OHdG: 8-hydroxy-2-deoxyguanosine
DNA damage: MN- micronuclei; SCE- Sister Chromatid Exchange
Anti-oxidant enzymes: SOD- superoxide dismutase, CAT- catalase, GPx- glutathione peroxidase, GR- glutathione reductase; GST- Glutathione S-Transferase
Other enzymes: G6PD- Glucose-6-phosphate dehydrogenase (required for production of NADPH, a co-enzyme for GR enzymatic activity)
Non-enzymatic antioxidant: GSH- glutathione, GSH/GSSG- ratio of reduced/oxidized glutathione, α-tocopherol, β-carotene, Se- selenium
SES- socio-economic status
‡values converted from µmol/L to µg/dL (0.48 µmol/L= 10 µg/dL)

Chapter 3

Blood lead levels and related socio-demographic predictors in school-age children from Montevideo, Uruguay

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ABSTRACT:

Elevated blood lead levels (BLLs) have been observed in preschool children from Montevideo, even after the removal of lead from gasoline, but the current extent of lead exposure in older children is unknown. For formulating effective prevention strategies, it is important to identify salient factors associated with childhood lead exposure in this population. The current study examined the prevalence and predictors of BLLs in 5 to 8 year old children living in different neighborhoods of Montevideo. The mean (SD) blood lead level was 4.7 (2.2) µg/dL, with 30.2% children having BLL ≥5 µg/dL, the current reference level set by the US Centre for Disease Control for identifying and monitoring children with elevated BLL. Father's occupation with potential for lead-exposure (such as construction, factory work, print shop, mechanic or driver), having more than one young siblings in the household and father's smoking were all associated with higher BLLs and having an elevated BLL (≥5 µg/dL). Because many studies show evidence of neurobehavioral deficits and other health problems in children and adults at the BLL of 5 µg/dL or lower and because recent risk assessments suggest that there is perhaps no safe level of lead exposure, these Uruguayan children are at risk for lead toxicity. Therefore, routine lead screening to identify at-risk children along with targeted interventions such as educational campaigns at school that focus on the reduction of risk factors for environmental exposure could be beneficial in this setting.

Keywords: lead, child, school-age, Uruguay, Latin America

1. INTRODUCTION:

Lead exposure in children continues to be an environmental health problem globally (Attina &Trasande 2013). Currently, the focus is mostly on low-level exposure rather than overt lead poisoning since the latter is rare in the general population. According to an estimation of the global burden of lead exposure conducted in 2004, about 40% of all children had blood a BLL above 5 µg/dL and 20% had levels above 10 µg/dL, with 90% of these children living in developing countries (Fewtrell et al., 2004). The concern with lead is that it contributes to a wide range of morbidities in children even at low level of exposure (ATSDR, 2007). Perhaps the most well-studied health effect is the neurotoxicity of lead, with research consistently showing cognitive deficits and behavioral problems at a progressively lower BLLs and no evidence of a threshold (Bellinger 2011; Koller et al 2004; Lanphear et al 2005; Canfield et al 2003; Kordas et al 2006; Braun et al 2006). The relationship between BLL and neurodevelopmental deficits is also non-linear with greater effects observed at lower levels of exposure. In many studies, lead exposure has been associated with increased risk for Attention Deficit Hyperactivity Disorder (ADHD), (Braun et al 2006; Froelich et al 2009; Nigg et al 2008, 2010), a behavioral disorder commonly seen in children. Moreover, early-life lead exposure has potential long-term effects on cognition and behavior well into adult years (Needleman et al 1996; Dietrich et al 2001; Wright et al 2008; Nevin, 2007; McCall et al 2004; Mazumder et al 2011).

Apart from the neuro-developmental deficits, other morbidities such as decreased growth (Schwartz et al 1986; Shukla et al 1991; Kafourou et al 1997; Ignasiak et al 2006), decreased hearing acuity (Schwartz and Otto 1987), higher incidence of dental carries (Moss et al 1999; Campbell et al 2000; Gemmel et al 2002) and delayed sexual maturation (Selevan et al 2003; Wu et al 2003; Hauser et al 2008; Williams et al 2010) in children have been related to lead

exposure. Given the impact that lead exposure has on children's health, especially on their neurobehavioral development, there should be continued effort to reduce lead exposure from children's environment.

While eliminating lead from the environment remains the primary prevention strategy, other behavioral and/or educational interventions targeted to the lead-exposed children and their families may provide a more practical solution in resource-poor settings. For the development of effective risk assessment, monitoring and prevention strategies, it is important to understand the environmental and socio-demographic factors related to lead exposure. The main known environmental sources of exposure have been the emissions from vehicles that use leaded fuels, water supplied through leaded pipes, lead-based paint in older housing, food prepared in lead-glazed ceramics, lead in soil and dust from leaded paint, gasoline, or past and present mining and industrial activities (Mielke 2002; Mielke and Reagan 1998). While lead has been withdrawn from gasoline in most countries around the world, exposure from other sources still continues (CDC 2005).

Children's lead exposure risk varies according to their age, proximity to the source, and certain behaviors like pica, hand- to-mouth and object-to- mouth activities (Lanphear et al 2002; Mielke and Reagan 1998). Children's blood lead concentrations typically peak during the first three years of life and then gradually decline (Dietrich et al 2001). Many studies reported that children whose parents are engaged in occupations with potential risk for exposure or parents engaged in activities like metal extraction, battery recycling or wire burning have higher blood lead levels (Liu et al 2012; Queirolo et al 2010; Brown et al 2005; Albalak et al 2003; Friedman et al 2005; Ruangkanchanasetr et al 2002). Studies consistently found an inverse association between socio-economic status (SES) and children's BLL (Disalvo et al 2009; Hussein et al

2008; Jiang et al 2010; Jones et al 2009; Naicker et al 2010; Queirolo et al 2010; Roy et al 2009; Ruangkanchanasetr et al 2002; Stark et al 1982; Ahmed et al 2010). Other socio-demographic factors like parental smoking behavior (Liu et al 2012; Nriagu et al 2011; Friedman et al 2005) and parental education (Liu et al 2012; Queirolo et al 2010; Nriagu et al 2011) were significant predictors in many studies. So far, research has identified some common socio-demographic risk factors, but there are several cultural and geographical differences. For example, race/ethnicity is an important risk factor in the US, with African American and Hispanic children being at greater risk for higher BLL (Kauffman et al 2000; Bernard et al 2003; Guttinger et al 2008; Raymond et al 2009). In Mexico, the use of lead-glazed pottery for preparing food has been identified as an important source of exposure (Olaiz et al 1996; Meneses-González 2003; Hernández-Serrato et al 2003). There have been reports that certain types of candies (Medlin 2004) and candy wrappers (Fuortes and Bauer 2000) produced in Mexico contain substantial amounts of lead and can be important sources of exposure (CDC 2002). "Kohl" applied to the eyes of children in South- East Asia, especially in countries such as India and Pakistan (Al-Saleh et al 1999; Rahbar et al 2002), and the use of herbal medicines are also considered important risk factors (Nriagu et al 2011; Mitchell et al 2012; Mitra et al 2012).

In Uruguay, lead was used in gasoline until 2004 (Mañay et al 2010). However, activities such as battery recycling, wire burning, particularly by low-income families, and the use of water from old leaded pipes continue to be possible sources of exposure (Mañay et al 2008). Although high levels of lead exposure have been reported in Uruguayan children (Schutz et al 1997; Cousillas et al 2005; Mañay et al 2008), it is perceived as a problem of low-income populations living in certain neighborhoods that have a history of lead poisoning. In 2010, Queirolo and colleagues reported on the prevalence of lead exposure in Uruguayan children aged 5 to 45

months [mean (SD) BLL: 9.0 (6.0) µg/dL] living in a variety of neighborhoods of Montevideo and identified important family and child-related predictors of BLL in this population. This study was among the first to provide evidence that lead exposure continues to be a problem in Uruguay even after the removal of lead from gasoline.

Here, we extend the study of the prevalence and predictors of blood lead levels to school age (5-8 years) children living in several neighborhoods of Montevideo. **Fig 2** depicts the hypothetical associations between the socio-demographic factors (for which we have information available) and BLL in these children. Since there is no routine blood lead testing of children in Uruguay, there is a lack of data on the extent of lead exposure in this age group. Identifying any at-risk groups and potential factors contributing to children's elevated blood lead concentrations may help focus future prevention and intervention efforts, including the communication of exposure risks.

2. METHODS:

2.1. Study setting and participant recruitment:

The current study was conducted in Montevideo, the capital of Uruguay from July 2009 to June 2011. Children who participated in the study attended private elementary schools and lived in Montevideo neighborhoods of nine municipal areas (**Fig 3**). Lead exposure was suspected or documented in these neighborhoods previously (Cousillas et al., 2005; Queirolo et al., 2010; Schutz et al., 1997).

The advertisement for the study was made via posters, radio, television, and newspaper announcements. In addition, all private elementary schools in the selected neighborhoods were identified from a list compiled by the Faculty of Psychology at the Catholic University of Uruguay, phonebooks, and private contacts. The schools in Montevideo are divided according to

the socioeconomic status (SES) of the students/families they serve. For the purpose of this study, directors of schools from low-to-middle SES were contacted. Letters of invitation were sent to the school directors, briefly explaining the study and asking for an in-person meeting to discuss the possibility of the school's participation. During the meeting with the directors, the procedures, risks, benefits and the duration of the study was explained in detail. Upon the director's agreement to participate, posters advertising the study were hung up at the school, and an informational meeting for the parents was scheduled. In total, 9 schools agreed to participate in the study.

The informational meetings were held at each school for the parents of all first grade children who had an interest in learning about the study. At the meetings, general information on childhood lead exposure such as common sources, routes of exposure, absorption, and health effects were provided. The rationale for the study and the study procedures were explained in detail. Questions from parents were answered and consent forms were provided. Parents had the option of providing their consent at the meeting or returning the signed forms after consulting with other family members.

All first grade children who regularly attended the participating schools were eligible for the study. The sole exclusion criterion was a previous diagnosis of lead poisoning (BLL >45 µg/dL). None of the children were excluded based on this criterion. Of the 410 eligible children from 9 private elementary schools, 211 agreed to participate and enrolled into the study. **Fig 4** shows both the overall and school-specific participation rate of first-grade children in the study. The study was approved by the Ethics Committee for Research Involving Human Participants at the Pennsylvania State University, the Catholic University of Uruguay, and the University of the Republic of Uruguay.

2.2. Parental questionnaire:

Parents/caregivers who agreed to participate in the study were invited for a meeting at the school to fill out a questionnaire about socio-demographic characteristics of the family, the child's medical history and the home environment. Briefly, caregivers were asked to provide detailed information about their age, education, occupation, smoking history, and family structure. To assess socio-economic condition and home environment of the families, caregivers were asked to fill out questions on their monthly income, daily expenditures on food and clothes, home ownership, number of rooms, number of persons living in the house, including number of children < 5 years of age; and family possessions of 12 household items like TV, video player, DVD player, computer, video games, radio, sound equipment, refrigerator, washer, home phone, cellular phone and car. In addition, the questionnaire consisted of queries regarding children's age, sex and medical history. Queries were made on the history of the mother's pregnancy and child birth. To identify possible environmental sources of exposure, information about the type of dwelling, frequency of dusting or cleaning the house, and sources of drinking and cooking water were enquired. The questionnaires were self-administered but the research staff provided assistance if parents or caregivers asked for clarification.

2.3. Home Observation for the Measurement of the Environment (HOME) Inventory:

The quality of the caretaking environment was evaluated by the HOME inventory for ages 6 to 10 years (Caldwell and Bradley, 2003). The inventory contains 59 items grouped into eight subscales—1) parental responsibility, 2) encouraging maturity, 3) emotional climate, 4) learning materials and opportunities, 5) active stimulation, 6) family participation, 7) parental involvement, and 8) physical environment. The Spanish translation of the original questionnaire, kindly provided by researchers from the National Institute of Perinatology in Mexico, was used

for the purpose of our study. The inventory was administered by a social worker who visited the child's home at a previously scheduled time. While completing some items in the inventory, the social worker made direct observations of the home and the child, for others, queries were directed to the caregivers. The interviewer's queries and direct observations were combined to produce scores on specific subscales, and subsequently the overall total score was derived by adding the scores on all 8 subscales.

2.4. Blood lead analysis:

Fasting blood was collected by a phlebotomy nurse at the school during a morning visit. Approximately 3 ml of venous blood was collected from each child using a 25-gauge safety butterfly blood collection set (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) in heparin coated trace-metal free tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The samples were stored on ice until the end of the clinic visit and then transported to the CEQUIMTOX, the Toxicology Laboratory in the Faculty of Chemistry at the University of the Republic (Montevideo, Uruguay). At the laboratory, whole blood tubes were stored at -20°C until analysis.

Blood lead concentrations were measured by Atomic Absorption Spectrometry (AAS, VARIAN SpectrAA-55B) using flame or graphite furnace ionization techniques, depending on the volume of whole blood available. The detection limit was 1.8 µg/dL for the flame and 0.8 µg/dL for graphite furnace AAS techniques. Analytical conditions were validated with standard quality assurance/ quality control (QA/QC) procedures (Parson and Chisolm, 1997). The laboratory participates in CDC's Lead and Multi-Element Proficiency Program and the Interlaboratory Program of Quality Control for Lead in Blood, Spain.

2.4. Statistical analysis:

2.4.1. Variable definitions:

Based on the answers provided in the parental questionnaire, mothers' occupations were divided into five categories: (1) unemployed, (2) domestic help, (3) factory work, (4) health work or police and (5) administration or professional work. Father's employment fell under six categories: (1) unemployed, (2) construction, (3) factory work/print shop, (4) mechanic, (5) security/military and (6) administrative/professional jobs. Father's occupation was also dichotomized as 1 if father was engaged in jobs with potential exposure to lead (construction, factory work, print shop, mechanic and driver) and 0 if otherwise. Additionally, parents were asked to indicate whether in the previous 6 months they performed jobs with potential for exposure to lead, such as jobs that involve chemicals or metals, recycling, auto-repair, plumbing and paint work for 6 months or more. The following coding scheme was applied to their responses: "0" if neither of the parents was involved, "1" if one of the parents was involved and "2" if both parents were involved. The reliability of the 14-item "occupational exposure" questionnaire was measured with Chronbach's alpha, yielding a statistic of 0.6. The total score was derived by adding the responses to all the items which ranged from 0 to 8. Because a large proportion of the parents (66.9%) reported no involvement in jobs listed in the questionnaire, total "occupational exposure" score was also collapsed into not-involved vs. involved. Parent's education was defined as having (1) some primary, (2) some secondary or (3) college education or higher.

The data on family's possession of 12 household items was collected as one of the measures of SES (Queirolo et al 2010). Parents indicated if the family possessed a particular item as "yes" or "no". All 12 items were entered into a factor analysis and a single factor with an Eigenvalue >1 was identified. We decided *a priori* to include in the final construction of a factor

only those items that had factor loadings greater than or equal to 0.3. Six items (video player, computer, car, refrigerator, washing machine and home phone) fit the above criterion. Scores for the 6 items were summed and then the total score (range: 0-6) was dichotomized at the average score of 3 items.

The family's living arrangements were categorized as 1) "living with both parents", 2) "living with one of the parents" or 3) "living with other people". Crowding was calculated as the number of persons living in the house divided by the number of rooms, and included as a categorical variable (less than equal to 2 people per room and greater than 2 people per room) in the analyses. Number of young children (< 5 years) in the household was categorized as having (1) no child less than 5 years or (2) one or more children less than 5 years. Previous studies have shown that having one or more siblings is a predictor of high blood lead levels (Liu et al 2012).

All the responses regarding potential environmental exposures within the home such as frequency of cleaning or dusting the house, presence of doormats at home, whether family members enter the house with shoes on, number of hours the windows are open in summer or winter, gardening in the house, and type of water used for cooking or drinking were categorized according to the potential severity of exposure they provided. For example, parents were asked to indicate how many times they clean the floors of the house, and the response options were: 1) "less than once per week", 2) "once per week" or 3) "more than once per week". For this analysis, the answers were reverse-coded to indicate that "more than once per week" likely reflected lower level of exposure. We also asked the parents to indicate the number of hours per day they kept the windows of the house open in summer and the choices were (1) never, (2) less than 1 hour, (3) 1-4 hours, (4) 5-12 hours and (5) more than 12 hours. Since more than half of the

households kept the windows opened for greater than 12 hours, the variable was dichotomized at 12.

Finally, study children lived in nine municipal areas or zones. Because some of the zones had very few participants, and because about 30% of the study children came from one particular municipal area (El Cerro) previously known for lead exposure, a dichotomous variable was created and differences in BLL among children living in that area vs others were tested.

2.4.3. Complete case analysis:

The main statistical tool used in this study was regression model. The primary outcome of the study was children's BLL. We built bivariate and multivariate linear and logistic regression models, with BLL (continuous and categorical) as the dependent variable and other variables as independent predictors. First, all the socio-demographic and environmental variables were tested individually in bivariate linear and logistic regression models. Next, the variables which were associated with BLL at $p< 0.25$ in bivariate analysis were entered into the multiple regression models. The multivariate models also included variables that have biological plausibility or were previously known to be related with children's BLL. Finally, the variables with $p< 0.1$ were retained in the multivariate model with the help of a backward stepwise variable deletion process.

2.4.3. Missing data- Multiple Imputations:

In the complete-case-analysis, the outcome variable of this study, BLL, and all the possible predictor variables except for sex and school were subject to missing data. Only 69 children (32.7%) had all the data on BLL and the predictor variables that were significantly associated with BLL in the bivariate regression analyses. To avoid potential bias and loss of power from complete-case analysis, multivariate multiple imputation (MI) was used to impute

missing values. The imputation model included the primary outcome variable (BLL), 13 predictor variables (variables that were significantly associated with BLL at $p< 0.25$ in bivariate regression model in the complete-case analysis), other biomarkers such as children's hair lead, arsenic, cadmium, manganese levels, hemoglobin (measured by portable hemoglobinometer; HemoCue, Lake Forest, CA), serum ferritin concentrations (measured by immunoradiometric assay, Coat-A-Count Ferritin IRMA; SIEMENS Diagnostic Products, USA) and other socio-demographic variables from all 211 children who completed the study. Imputation by chained equations (command "mi ice") of STATA version 12.0 (StataCorp, College Station, TX) was conducted (Royston and White, 2011).

Briefly, the algorithm of "mi ice" works in the following manner: first, each variable with missing values is regressed on the other variables in the imputation model. Next, missing values in each variable are replaced by simulated draws from the posterior predictive distribution of the variable derived from the regression estimation. The process is repeated for all the incomplete variables in the imputation model and the entire round is called a cycle. To stabilize the results, the regression switching cycle is iterated 10 times (default in STATA) and finally a single imputed dataset is generated. We created 10 imputed datasets for our analyses.

2.4.4. Final Model selection with imputed data:

The selection of a parsimonious model for BLL was performed using the imputed datasets. First, a multiple linear regression model was fitted with BLL as outcome and all the eligible variables as predictors. The models were fitted to all the imputed datasets and the results of each individual imputed dataset were combined using Rubin's rules (Royston, 2004) by STATA. Then, variables with $p< 0.1$ were manually removed stepwise in a backward fashion to establish the final predictive model.

3. RESULTS:

3.1. Socio-demographic characteristics of the study children:

The summary of the biological, parental and socio-economic characteristics of the study children are presented in **Table 5** and are derived from the non-imputed data. Among 211 enrolled children, 189 attended the biological sample collection and anthropometric assessment sessions at school and 182 provided blood samples for measurement of lead concentration. Blood from seven children who attended the sessions could not be collected mostly because they did not cooperate or denied to provide oral consent to draw blood. The average age of the study children was 6.8 (range: 4.7-8.3) years. Children had a mean (SD) blood lead level (BLL) of 4.7 (2.2) µg/dL, with 30.2% children having an elevated BLL (≥ 5 µg/dL). There were no significant sex differences in BLL among these children. More than half of the parents had some secondary education, and children whose fathers attended college or had some post-graduate education had significantly lower BLL than children who had fathers with only primary education (3.6 ± 1.2 µg/dL vs 4.6 ± 2.3 µg/dL; $p< 0.05$) (Table 5). About 38.3% of the mothers were unemployed/ stayed at home, whereas 9.1% were engaged in factory jobs. More than half of the fathers were engaged in jobs with potential for lead exposure. Children who had fathers with potential for exposure at work had significantly higher BLL than children whose fathers were not at risk for exposure (5.1 ± 2.3 µg/dL vs 4.1 ± 1.7 µg/dL; $p< 0.05$). Similarly, children whose fathers smoked (41.8%) had higher BLL than those whose fathers did not smoke (5.2 ± 2.7 µg/dL vs 4.4 ± 1.8 µg/dL; $p< 0.05$). The mean (SD) score on HOME index was 43.3 (9.3), with more than half of the children having less than median score of 46. About 23% children lived in crowded households (> 2 person/room) and had higher mean BLL than children who did not have crowding at home ($p< 0.05$). About 65% of the families had one or more young child (< 5 y old)

in the household. Families with very young children had first-graders with higher BLLs than families without very young children (5.5 ± 2.4 vs 4.4 ± 2.0 µg/dL; $p< 0.05$). Finally, children with parents living together without marriage had higher BLL than those with married parents (5.1 ± 2.3 µg/dL vs 4.1 ± 1.6 µg/dL; $p< 0.05$).

Table 6 presents children's age, sex, and other socio-demographic characteristics (such as mother's age, parental education, smoking status, home ownership, crowding, family possessions of household items) by schools. They did not differ significantly. Moreover, there were no differences in children's BLL according to neighborhood (**Table 7**). However, when one particular area (El Cerro) was compared with other areas, children living in El Cerro had significantly higher mean BLL than children living in other areas (5.5 ± 2.6 µg/dL vs 4.3 ± 1.9 µg/dL; $p< 0.05$) (Table 7).

In bivariate regression models, 13 variables were associated with children's BLLs at $p< 0.25$. The variables that had an association with BLL at $p< 0.1$ are presented in **Table 8**. For example, mother's age was inversely associated with children's BLLs (β= -0.06; 95% CI: -0.11, -0.01) and there was a slightly lower likelihood of children having elevated BLLs for an increase in mother's age [Odds Ratio (OR): 0.93; 95% CI: 0.88, 0.98] in bivariate regression models (Table 4). Fathers with any college or post-graduate education had children with 75% lower risk of having elevated BLLs than fathers with primary education. Father's job with potential lead exposure such as construction, factory work, print shop, mechanic and driver was associated with higher BLLs (β= 1.05; 95% CI: 0.35, 1.74) and with a 3.1- fold higher likelihood (95% CI: 1.46, 6.69) of elevated BLLs than father's jobs without potential for exposure. Mothers who were engaged in administrative or professional work had children with 90% lower likelihood of elevated BLLs than mothers who were unemployed/stayed at home. Children had 1 µg/dL higher

BLL (β= 0.97; 95% CI: 0.19, 1.74) and a 2.9-times higher likelihood of having BLLs ≥ 5 µg/dL (95% CI: 1.23, 6.68) if their parents were living together than if they were married. Fathers who smoked had children with 0.87 µg/dL higher BLL (95% CI: 0.11, 1.62) and a 2.4- fold higher likelihood of elevated BLLs (95% CI: 1.19, 5.02). Having young children (< 5 years) in the household was a significant predictor of BLL (β= 1.11; 95% CI: 0.39, 1.83) and there was a higher likelihood of having children with elevated BLL (OR: 3.10; 95% CI: 1.52, 6.32) if one or more young child was present in the household. The HOME Inventory score was inversely associated with BLL (β= -0.06 µg/dL; 95% CI: -0.1, -0.02) (Table 8).

3.2. Household behaviors and differences in blood lead concentration:

Table 9 presents the data on household behaviors and how children's BLLs differ according to these factors. Approximately 52.1% of the families kept the windows of their house open for more than 12 hours. Children in families that kept their windows opened for more than 12 hours during summer had slightly higher BLLs than those who opened their windows for less time (4.4 ± 2.1 µg/dL vs 4.9 ± 2.2 µg/dL; p< 0.1) (Table 9). About 72% and 60% of the families reported using filtered water for drinking and cooking, respectively. Although there were differences in mean BLL among children whose families used filtered water for drinking and cooking than those who did not, the difference did not reach statistical significance. A majority (79.6%) of the families reported that they cleaned the floors of their house more than once in a week compared to 3.6% of the families who cleaned their floors less frequently. No significant difference in children's BLL was found between these families (Table 9). In bivariate regression models, there was a slight positive association between the number of hours the windows were opened in the summer and children's BLLs (β= 0.52; 95% CI: -0.16, 1.20) (Table 8).

3.3. Multivariate complete-case analyses:

We conducted multiple linear and logistic regression models with the socio-demographic and household variables to understand the strongest predictors of BLLs among these two sets of parameters. In multivariate complete-case analyses, the most salient socio-demographic predictors of children's BLLs were father's job exposure risk and having at least one very young child in the household (**Table 10**). There was a 1 µg/dL higher BLL (95% CI: 0.37 – 1.67) and 3.4 times higher likelihood of children having an elevated BLLs (BLL≥ 5 µg/dL) (95% CI: 1.44 – 8.13) if fathers had potential lead-exposure risk at their jobs than fathers who did not have exposure risk at work. Families with one or more very young children had higher BLLs (β= 0.80; 95% CI: 0.12 – 1.48) and higher likelihood (OR: 2.76; 95% CI: 1.19 – 6.37) of having elevated BLLs than families without very young children.

While father's smoking was the strongest household behavior predicting children's blood lead levels or a child having an elevated BLL in multivariate models, windows open for more than 12 hours in the summer and drinking unfiltered water also marginally predicted blood lead concentrations in these children (**Table 11**). Fathers who smoked had children with 0.8 µg/dL higher BLL (95% CI: 0.05 - 1.59) and 2.5 times higher odds of children having elevated BLLs (95% CI: 1.19 - 5.36) (Table 11).

3.4. Multivariate analyses with imputed data set:

Except for child's sex and school, information was missing on all the socio-demographic and biological variables including the outcome variable (BLL), 13 predictors (variables that were significantly associated with BLL at p< 0.25 in bivariate regression models in the complete-case analysis), other biomarkers such as children's hair lead, arsenic, cadmium, manganese levels, hemoglobin, serum ferritin concentrations and other socio-demographic variables. When the pattern of missingness was tested, the variables were found to be missing completely at random

(MCAR). In other words, the probability of a missing observation for a given variable was completely independent of both the observed and unobserved values of other variables in the data set. We included 25 variables with missing data to build the imputation model. **Table 12** shows the levels of missingness of the variables included in the imputation model. To note, there is no theoretical guideline or consensus on how much missing data can be imputed on outcome, predictors or covariates. However, it is common practice to impute variables with 10 - 50% missingness.

Finally, when we repeated the multivariate regression models with the imputed dataset, we found similar results as in the complete-case analysis (**Tables 13 & 14**).

4. DISCUSSION:

The current report is one of the few studies on the prevalence and predictors of blood lead levels in Uruguayan children. With a mean (SD) of 4.7 (2.2) µg/dL and about 30% children having BLL≥ 5 µg/dL, the current reference level set by Center for Disease Control (CDC 2012) for identifying and monitoring children with elevated BLL, the extent of lead exposure in these 5-8 year olds is lower than the previously reported estimates in the preschoolers living in and around the same neighborhoods of Montevideo. The mean (SD) BLL in the preschool-age children was 9.0 (6.0) µg/dL, with more than 30% of the children having BLL> 10 µg/dL (Queirolo et al 2010). This finding is not surprising since children's blood lead concentration typically peaks during the first 3 years of life, before gradually declining (Dietrich et al 2001). Furthermore, the current study was conducted a few years after the study in preschoolers and a lower trend in BLL is expected in studies conducted further away in time from the year that lead was removed from the gasoline (Pirkle et al 1998). However, the BLLs in this study were comparable with BLLs in children of the same age group from other Latin American countries.

For example, in 5-9 year olds from Cartagena, Colombia, the mean (SD) BLL was 5.5 (0.23) µg/dL, with 11.6% having BLL> 5 µg/dL (Olivero-Verbal et al 2004). Children (5-16 years old) from Posada, Argentina had a mean (SD) BLL of 5.8 (4.3) µg/dL, with 16% of the children having BLL≥ 10 µg/dL (Beltramino et al 2007). According to a 2009 report, the mean (SD) BLL of 2-8 years old children from different towns of Belize was 4.9 (2.5) µg/dL (Charalambous et al 2009). Given the concerns over the detrimental health effects of lead even at lower levels (BLL< 5 µg/dL), the estimates of lead exposure in children from Montevideo underscore the continued importance of this public health problem.

Father's employment in jobs with potential exposure risk (such as construction, factory work, print shop, mechanic and driver) and having more than one young child (< 5 year) in the household were significant predictors of blood lead level and of the likelihood of a child having a BLL≥ 5 µg/dL. Several studies, including the one in Montevideo preschoolers, have reported that parents' occupation is highly associated with children's elevated blood lead levels (Queirolo et al 2010, Liu et al 2012, Brown et al 2005, Friedman et al 2005, Albalak et al 2003, Rahbar et al 2002, Ruangkanchanasetr et al 2002). We found 3.6 times higher odds of having a BLL≥ 5 µg/dL in children whose fathers worked in an occupation with potential for lead exposure than children whose fathers were not employed in such jobs. Similar to our finding, Friedman and colleagues (2005) reported a 2.3 times higher likelihood of having a BLL> 4.65 µg/dL in Ukranian children whose fathers worked in jobs such as construction, steel and manufacturing industries compared to those whose fathers were employed in administrative or professional work. The fathers in our study who had the potential for occupational exposure worked in construction, factories, and print shops, and as mechanics or drivers. It is possible that dust

contaminated by lead settled on fathers' skin and clothing, and later served as sources of exposure to children at home (Levin et al 2008).

Father's job exposure risk may also reflect the socio-economic status (SES) of the family. Fathers with high SES are less likely to be involved in jobs/behaviors that will put them at risk of lead exposure. Families with better resources may be more likely to live in areas with fewer industries, have higher parental education (and therefore more awareness of health risks), have better housing conditions, and be engaged in behaviors protective of child health (better nutrition and hygiene, for example) (Queirolo et al 2010; Hussein et al 2008; Chen et al 2003). In this study, fathers engaged in jobs with potential for lead exposure had families with lower incomes and slightly higher crowding than those who were not employed in such jobs (income: 11,347 ± 6,928 vs 17,308 ± 16,187 Uruguyan pesos; crowding: 2.1 ± 0.9 vs 1.8 ± 0.6 people/room; $p< 0.05$). In turn, study children from families with better SES (income≥ 10,000 Uruguayan pesos; crowding< 2 people/room) experienced a slightly lower mean BLL of 4.5 µg/dL as compared to children (BLL of 5.2 µg/dL, $p< 0.1$) with lower family income (less than 10,000 Uruguayan pesos) and more crowding at home (greater than 2 people/room). While about 25% of children with better family SES had elevated BLLs (≥ 5 µg/dL), the prevalence (45%) was higher among children with lower family SES ($p< 0.05$).

The number of young children in a household was also a significant predictor of BLLs in first-graders. This is consistent with other studies, which reported that having siblings was a predictor of higher blood lead levels (Liu et al 2012; Jarosinska et al 2004, Cikrt et al 1997, Osman et al 1998, Jain et al 2006). For example, Liu and colleagues (2012) observed higher BLL in Chinese children with one or more siblings than children without any siblings. It is possible that older children play with the young siblings outdoors or on the floor and get exposed to lead

from dust or soil (Liu et al 2012). On the other hand, having young children in a household may also affect the socio-economic conditions of the family, with more resources needing to be allocated to their care. We observed that households with young children had younger mothers (31.0 ± 7.0 vs 34.0 ± 6.5 years; $p< 0.05$), lower maternal education (secondary: 50.5% vs 55.2%; college or post-graduate: 13.8% vs 36.7%; $p< 0.05$) and more crowding (2.4 ± 1.0 people/room vs 1.7 ± 0.5 people/room; $p< 0.05$) than households without young children. Larger families may also be poorer and have exposure risks associated with lower SES.

Among the household behaviors, father's smoking was the strongest determinant of children's blood lead level. This is consistent with findings by others (Liu et al 2012, Apostolou et al 2011, Ordonez Iriate et al 2009, Friedman et al 2005). In a recent study, Apostolou et al (2011) showed that living with one or more smokers was associated with higher blood lead levels among US children. There was also an association between children's cotinine levels and BLLs (Apostolou et al 2011). In our study, although the relation between mothers' smoking and children's BLLs did not reach statistical significance, the trend was similar to that of the father's smoking. The proportion of smokers among mothers (34%) was not very different than that of the fathers (40%). Among the fathers who reported smoking, 42% had spouses/partners who smoked as well. Tobacco smoke contains lead (Galazyn-Sidorczuk et al 2008; Kalcher et al 1993), which can be inhaled and absorbed through the lungs (ATSDR, 2007). We do not have information on whether the parents smoked in the home or outside, how many hours the parent spent at home, the ventilation in the house or the cotinine levels of these children. However, the majority of the parents were either married or living together and 70% of the study children lived with their parents. So, exposure to second-hand smoke at home is very likely for these children.

Children living in one particular municipal area (El Cerro) had higher average BLL than the average BLL of children living in other areas combined. Anecdotally, this area is surrounded by garment and paint industries, which may be potential sources of exposure to lead in children. We collected geographical information and environmental samples from children's dwellings. In future, analyses of these data could provide more insight into the exposure characteristics and geospatial differences in children's BLL in Montevideo.

Although we attempted to test and identify as many common socio-demographic and environmental predictors of BLLs in the first-grade children as possible, there may have been other significant predictors in this population that were not captured by our study (**Fig 5**). These include distance of the child's house or school from road traffic, use of lead-based paints for painting the house, housing condition, use of herbal/traditional medicines or glazed ceramic bowls in the household, personal hygiene, and certain behaviors like pica or chewing/sucking colored pencils. Furthermore, unlike the study in Montevideo pre-schoolers, we failed to find significant associations between BLL and variables that measure SES (such as income, family possessions, owning a house, or crowding). However, we did observe a higher risk of elevated BLL (≥ 5 μg/dL) in children who lived in a crowded (> 2 people/room) household. We did a purposeful sampling by choosing children from low-to-middle income families, thus there may be less variability in socio-economic status of these families than in the general population. In addition, many parents (52%) did not provide information on their income, which may have resulted in a null association between income and BLLs due to loss of power. However, we used multiple imputations to handle the problem of missing data and our results were similar in both complete-case and imputed models. Finally, the results on environmental factors were based on a self-reported parental questionnaire and thus potentially subject to response bias.

Despite these limitations, ours is one of the few studies in Uruguay and thus provides a unique opportunity to understand the extent of childhood lead exposure and the factors that are predictive of lead levels in first-grade children of this country several years after the removal of lead from gasoline. Although other studies have identified factors that can affect children's lead exposure, country or culture-specific differences exist. Identification of the predictors of lead exposure in this population may help in formulating policy, initiating preventive measures or targeting intervention programs. Furthermore, the participants of this study were comprised of first-grade (5 to 8 y) children. While our result cannot be generalized to children of other age groups, this provides a unique age-specific data for children at school entry. Many studies evaluated a wide age range of children and did not tease out the differences in factors that predict children's BLLs according to age (Anticona et al 2012; Ahamed et al 2010; Disalvo et al 2009). However, age-specific differences in BLLs or in predictors of BLL do exist (Anticona et al 2012; Mitchell et al 2012). For example, children's BLLs normally decrease as they age and hand-to-mouth or object-to-mouth activities are more prevalent among young children than older ones (Dietrich et al 2001; Lanphear et al 2002). On the other hand, school children are likely to spend a substantial amount of their time at school, therefore the location or environment of the school becomes an important factor (Albalak et al 2003). In addition, school entry is an important time for the study of lead exposure because it is a time of enormous social, behavioral and cognitive growth, all of which may be affected by lead.

5. CONCLUSION:

In summary, we found evidence of continued lead exposure in Uruguayan children even after the removal of lead from gasoline. The blood lead values in these school-age children were at a level that could put them at risk for cognitive and behavioral deficits, which may in turn affect their academic achievement and behavior. Therefore, lead screening should be made a routine practice, and school entry appears to be an opportune time to carry out the testing, if no previous screenings were done. It could be combined with other routine screening tests of importance to school achievement including vision and hearing.

Our study also identified factors such as father's smoking, job exposure risk and having young children in the household as significant predictors of blood lead levels in these children. Some of these sources/behaviors of lead exposure could be modifiable and potential targets for interventions. Efforts such as promoting a smoke-free environment, increasing awareness at the work-place to reduce take-home exposure, educating parents, teachers and children to change behaviors and life-style could be initiated by health-care providers, public-health specialists, educators and the government. School entry is an excellent time to implement educational campaigns around the reduction of risk factors for environmental exposure. Such campaigns may be beneficial to the children themselves as well as their siblings.

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Table 5: Socio-demographic characteristics of Uruguayan first graders

	N	Mean	SD	Range	%	BLL ± SD (µg/dL)
Biological factors						
Age (y)	211	6.8	0.6	4.7 – 8.3	---	---
Sex	211	---	---	---	---	---
Girls	---	---	---	---	43.1	5.0 ± 2.4
Boys	---	---	---	---	57.7	4.5 ± 1.9
Blood lead levels (BLL) µg/dL	182	4.7	2.2	0.8 – 13.2	---	---
≥ 10 µg/dL	---	---	---	---	3.3	---
≥ 5 µg/dL	---	---	---	---	30.2	---
Parental factors						
Mother's age	184	33.5	6.8	21 - 57	---	---
Mother's education	183	---	---	---	---	---
Some primary	---	---	---	---	20.2	4.8 ± 2.3
Some secondary	---	---	---	---	65.6	4.8 ± 2.1
College or post-grad	---	---	---	---	14.2	4.3 ± 2.3
Mother's occupation	154	---	---	---	---	---
Unemployed	---	---	---	---	38.3	4.8 ± 2.2
Domestic help	---	---	---	---	33.8	4.6 ± 2.3
Factory work	---	---	---	---	9.1	5.7 ± 2.5
Health work or police	---	---	---	---	6.5	5.3 ± 3.3
Administration or professional work	---	---	---	---	12.3	4.0 ± 1.1
Mother's smoking status	163	---	---	---	---	---
No	---	---	---	---	64.4	4.6 ± 2.3
Yes	---	---	---	---	35.6	4.9 ± 2.1
Father's education	148	---	---	---	---	---
Some primary	---	---	---	---	26.4	4.6 ± 2.3
Some secondary	---	---	---	---	55.4	5.1 ± 2.3
College or post-grad	---	---	---	---	18.2	3.6 ± 1.2**[a]
Father's job exposure risk[b]	141	---	---	---	---	---
No	---	---	---	---	48.2	4.1 ± 1.7
Yes	---	---	---	---	51.8	5.1 ± 2.4***
Father's smoking status	141	---	---	---	---	---
No	---	---	---	---	58.2	4.4 ± 1.8
Yes	---	---	---	---	41.8	5.2 ± 2.7**
Marital status	140	---	---	---	---	---
Married	---	---	---	---	43.6	4.1 ± 1.6
Divorced	---	---	---	---	18.6	4.8 ± 2.6
Living together	---	---	---	---	37.9	5.1 ± 2.3**
Child lives with	162	---	---	---	---	---
Both parents	---	---	---	---	69.1	4.6 ± 2.0
Single parent/others[c]	---	---	---	---	30.9	4.9 ± 2.6
Parental occupational exposure score[d]	194	0.5	1.1	0 – 8	---	---
No involvement	---	---	---	---	66.9	4.6 ± 2.3
Yes	---	---	---	---	33.1	4.9 ± 2.0
Socio-economic status and home environment						
Home ownership	165	---	---	---	---	---
No	---	---	---	---	61.0	4.7 ± 2.1
Yes	---	---	---	---	39.0	4.7 ± 2.3
Crowding (no of person/ no of rooms)	164	2.0	0.8	1 - 5	---	---
≤ 2 person/room	---	---	---	---	77.4	4.5 ± 2.2
> 2 person/room	---	---	---	---	22.6	5.2 ± 2.2**
Children < 5 y present at home	155	---	---	0 – 3	---	---
No	---	---	---	---	34.8	4.4 ± 2.0
Yes	---	---	---	---	65.2	5.5 ± 2.4**
Family possessions (6 household items)[e]	166	3.6	1.4	0 – 6	---	---
≤ 3 items	---	---	---	---	42.8	4.8 ± 2.3
> 3 items	---	---	---	---	57.2	4.6 ± 2.1
HOME Inventory score	131	43.3	9.3	12 – 56	---	---

[a]compared to children with dad having primary education. [b]has exposure risk if employed in jobs such as construction, factory work, print shop, mechanic and driver [c]others include grandparents, maternal or paternal uncles or aunts.
[d]involvement in activities that require handling of chemicals or metals, recycling, auto-repair, plumbing and paint work for 6 months or more; score derived by adding all the responses (0= none of the parents, 1= one of the parents, 2= both parents involved); no involvement if score=0 and involvement if score ≥1 [e]items include video player, computer, car, refrigerator, washing machine and home phone.
***p< 0.01 **p< 0.05 *p< 0.1

Table 6: Children's socio-demographic characteristics by school

Socio-demographic factors	Schools								
	School 1 (n= 12)	School 2 (n= 45)	School 3 (n= 14)	School 4 (n= 19)	School 5 (n= 28)	School 6 (n= 2)	School 7 (n= 3)	School 8 (n= 7)	School 9 (n= 81)
	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %
Children's age (y)	6.5 ± 0.3	6.7 ± 0.6	6.9 ± 0.6	6.7 ± 0.4	6.9 ± 0.6	7.2 ± 0.1	6.7 ± 0.3	6.7 ± 0.6	6.9 ± 0.5
Girls (%)	33.3	48.9	42.9	47.4	53.6	50.0	66.7	28.6	37.0
Boys (%)	66.7	51.1	57.1	52.6	46.4	50.0	33.3	71.4	63.0
Mother's age (y)	34.7 ± 7.2	32.9 ± 8.1	37.2 ± 6.4	33.0 ± 5.2	35.2 ± 6.5	43.0 ± 2.8	30.3 ± 7.7	35.2 ± 6.9	32.5 ± 6.2
Mother's education (%)									
Some primary	8.3	20.0	10.0	12.5	11.5	0.0	0.0	0.0	31.9
Some secondary	58.3	47.5	30.0	31.3	73.1	50.0	0.0	80.0	58.0
College/post-grad	33.3	32.5	60.0	56.2	15.4	50.0	100.0	20.0	10.1
Mothers smoke	33.3	39.0	30.0	33.3	26.9	0.0	33.3	40.0	37.3
Father's education (%)									
Some primary	16.7	21.6	0.0	6.7	29.2	0.0	0.0	20.0	46.4
Some secondary	58.3	67.6	20.0	53.3	62.5	50.0	66.7	60.0	48.2
College/post-grad	25.0	10.8	80.0	40.0	8.3	50.0	33.3	20.0	5.4
Fathers with job exposure risk (%)	41.7	51.5	22.2	53.3	39.1	0.0	33.3	40.0	54.6
Fathers current smoker (%)	41.7	46.9	0.0	46.7	34.8	50.0	0.0	40.0	47.3

Table 6 continued

Socio-demographic factors	Schools								
	School 1 (n= 12)	School 2 (n= 45)	School 3 (n= 14)	School 4 (n= 19)	School 5 (n= 28)	School 6 (n= 2)	School 7 (n= 3)	School 8 (n= 7)	School 9 (n= 81)
	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %	Mean ± SD or %
Home ownership (%)	41.7	53.7	80.0	62.5	55.6	50.0	33.3	20.0	72.7
Crowding (>2 people/room) (%)	8.3	24.4	0.0	18.7	38.5	0.0	0.0	25.0	25.0
Families with children <5 y (%)	41.7	34.2	10.0	26.7	26.9	0.0	33.3	20.0	46.5
Family possessions (6 household items) (%)									
≤ 3 items	25.0	51.2	10.0	18.7	59.3	0.0	33.3	20.0	48.5
> 3 items	75.0	48.8	90.0	81.3	40.7	100.0	66.7	80.0	51.5
HOME inventory score	44.5 ± 7.1	40.3 ± 10.2	50.6 ± 4.6	48.2 ± 5.3	46.4 ± 6.1	40.0 ± 0.1	45.5 ± 0.7	---[†]	39.3 ± 10.3

[†]data not available

Table 7: Differences in blood lead levels by municipal areas[a]

	N	% of children	BLL ± SD (µg/dL)
Neighborhood[b]	211		
1		20.0	4.7 ± 1.3
2		37.1	4.4 ± 2.2
3		42.9	4.9 ± 2.4
Municipal areas[c]	211		
El Cerro		30.5	5.5 ± 2.6
Others		69.5	4.3 ± 1.9**

**p< 0.05

[a]study children lived in nine municipal areas

[b]variable "neighborhood" created by collapsing nine municipal areas into three, according to their proximity

[c]municipal areas dichotomized into one municipal area (El Cerro)

Table 8: Unadjusted associations between socio-demographic factors, household behaviors, and BLL[1]

	BLL (µg/dL)[2]	**BLL ≥ 5 µg/dL[3]**
	β [95% CI]	OR [95% CI]
Mom's age	-0.06 [-0.11, -0.01]**	0.93 [0.88, 0.98]***
Fathers with college or post-graduate[b]	-1.05 [-2.10, -0.003]**	0.25 [0.06, .98]**
Father's job exposure risk	1.05 [0.35, 1.74]***	3.12 [1.46, 6.69]***
Mother's occupation		
Unemployed	Ref group	Ref group
Domestic help	-0.3 [-1.1 – 0.6]	0.8 [0.3 -1.6]
Factory work	0.8 [-0.5 – 2.1]	1.2 [0.4 – 3.8]
Health work or police	0.5 [-1.1 – 2.0]	0.7 [0.2 -2.9]
Administration or professional work	-0.9 [-2.0 – 0.3]	0.1 [.01 – 0.7]**
Parents living together[c]	0.97 [0.19, 1.74]**	2.90 [1.23, 6.68]**
Father smokes	0.87 [0.11, 1.62]**	2.44 [1.19, 5.02]**
Families with ≥ 1 children less than 5 y	1.11 [0.39, 1.83]***	3.10 [1.52, 6.32]***
HOME inventory score	-0.06 [-0.10, -0.02]**	0.95 [0.91, 0.98]**
Crowding (> 2 people/room)	0.71 [-0.10, 1.53]*	2.42 [1.13, 5.17]**
Windows open >12 hours in a household in summer	0.52 [-1.16, 1.20]*	1.58 [0.81, 3.11]

[1]complete-case
[2]variables tested in bivariate linear regression model
[3]variables tested in bivariate logistic regression model
[b]compared to fathers with primary education
[c]compared to parents who were married
*p< 0.1 **p< 0.05 ***p< 0.01

Table 9: Differences in blood lead levels by household behaviors[a]

Variables	N	%	BLL ± SD (μg/dL)
Window open in summer	163		
≤ 12 hours/day		47.9	4.4 ± 2.1
> 12 hours/day		52.1	4.9 ± 2.2*
Window open in winter	165		
≤ 4 hour/day		90.9	4.8 ± 2.4
> 4 hour/day		9.1	4.0 ± 1.8
Water for drinking	178		
Filtered		71.9	4.6 ± 2.1
Not-filtered		28.1	5.0 ± 2.5
Water for cooking	178		
Filtered		40.4	4.5 ± 2.0
Not-filtered		59.6	4.8 ± 2.3
Presence of vegetable garden in the house	163		
Yes		6.7	5.3 ± 1.8
No		93.3	4.7 ± 2.2
Frequency of floor cleaning per week	167		
More than once		79.6	4.6 ± 2.2
Once		16.8	4.7 ± 2.4
Less than once		3.6	5.2 ± 2.4
Frequency of dust cleaning per week	160		
More than once		55.0	4.7 ± 2.4
Once		35.0	4.7 ± 2.0
Less than once		10.0	4.2 ± 1.9
Presence of door-mats at the door	158		
No		51.3	4.6 ± 2.4
All or some		48.7	4.7 ± 2.0

*p< 0.1

[a]based on self-reported responses

Table 10: Multivariate associations between socio-demographic factors and blood lead level in Uruguayan school-age children[1]

	BLL	BLL ≥ 5 µg/dL
	β [95% CI][2]	OR [95% CI][3]
Father's job exposure risk	1.09 [0.42, 1.77]***	3.58 [1.44 – 8.88]***
Having ≥ 1 young child in a household	0.73 [0.01, 1.44]**	3.30 [1.09 – 6.39]***

[1]complete-case; n=133
[2]predictors tested and included in the final multivariate linear regression models; R^2 of the model= 26.2
[3]predictors tested and included in the final multivariate logistic regression model
[a]other schools as reference group
**p< 0.05 ***p< 0.01

Table 11: Multivariate associations of blood lead concentration with household behaviors in Uruguayan school-age children[1]

	BLL	BLL ≥ 5 µg/dL
	β [95% CI][2]	OR [95% CI][3]
Father smokes	0.82 [0.05, 1.59]**	2.52 [1.19, 5.36]**
Windows open more than 12 hours/day in the summer	0.74 [-0.03,1.50]*	2.05 [0.95, 4.40]*
Drinking un-filtered water	0.71[-0.16, 1.57][#]	0.98 [0.42, 2.33]

[1]Complete-case, multivariate analysis; n= 134
[2]predictors tested and included in the final multivariate regression model; R^2 of the model= 10.0
[3]predictors tested in the multiple logistic regression model
[#]p= 0.1 *p< 0.1 **p< 0.05

Table 12: Missingness of data on variables included in the multiple imputation model related to blood lead level of Uruguayan first-grade children[1]

Variables	No. of children with missing observations	% of children with missing observations
Child's age	2	0.9
Child's sex	0	0.0
Mother's age	27	12.8
Mother's smoking	30	14.2
Mother's education	28	13.3
Father's education	47	22.3
Father's smoking	54	25.6
Father's job exposure risk[2]	54	25.6
Crowing at home	29	13.7
Family possessions (6 household items)	27	12.8
Home ownership	29	13.7
Children <5 y present at home	42	19.9
Parent's marital status	55	26.1
HOME inventory score	80	37.9
Number of hours windows open in summer	31	14.7
Types of drinking water	33	15.6
Child's blood lead level	29	13.7
Hemoglobin	22	10.4
Serum ferritin	35	16.6
Serum C-Reactive Protein	37	17.5
Hair lead concentrations	25	11.8
Hair arsenic concentrations	25	11.8
Hair cadmium concentrations	25	11.8
Hair manganese concentrations	25	11.8

[1]Total number of children= 211

Table 13: Socio-demographic factors as predictors of blood lead levels (BLLs) and elevated blood lead level (BLL≥ 5 µg/dL) in Uruguayan school-age children based on imputed data sets[1]

	BLL	BLL ≥ 5 µg/dL
	β [95% CI][2]	OR [95% CI][3]
Father's job exposure risk	0.90 [0.23, 1.57]***	3.08 [1.34 – 7.08]**
Having ≥ 1 young child in a household	0.86 [0.18, 1.54]**	3.30 [1.40 – 7.50]***

[1]multiple imputed datasets (m= 10) used for analysis
[2]predictors tested and included in the final multivariate regression model
[3]predictors tested in the multiple logistic regression model
[a]compared to other schools
**p< 0.05 ***p< 0.01

Table 14: Multivariate associations of blood lead concentration with household behaviors in Uruguayan school-age children based on imputed data sets[1]

	BLL	BLL ≥ 5 µg/dL
	β [95% CI][2]	OR [95% CI][3]
Father smokes	0.75 [0.10,1.42]**	2.44 [1.20, 4.94]**
Windows open more than 12 hours/day in the summer	0.57 [-0.14, 1.27][#]	1.74 [0.85, 3.55]
Drinking un-filtered water	0.45 [-0.25, 1.18]	0.99 [0.46, 2.13]

[1]multiple imputed datasets (m= 10) used for analysis
[2]predictors tested and included in the final multivariate regression model
[3]predictors tested in the multiple logistic regression model
[#]p= 0.1 *p< 0.1 **p< 0.05

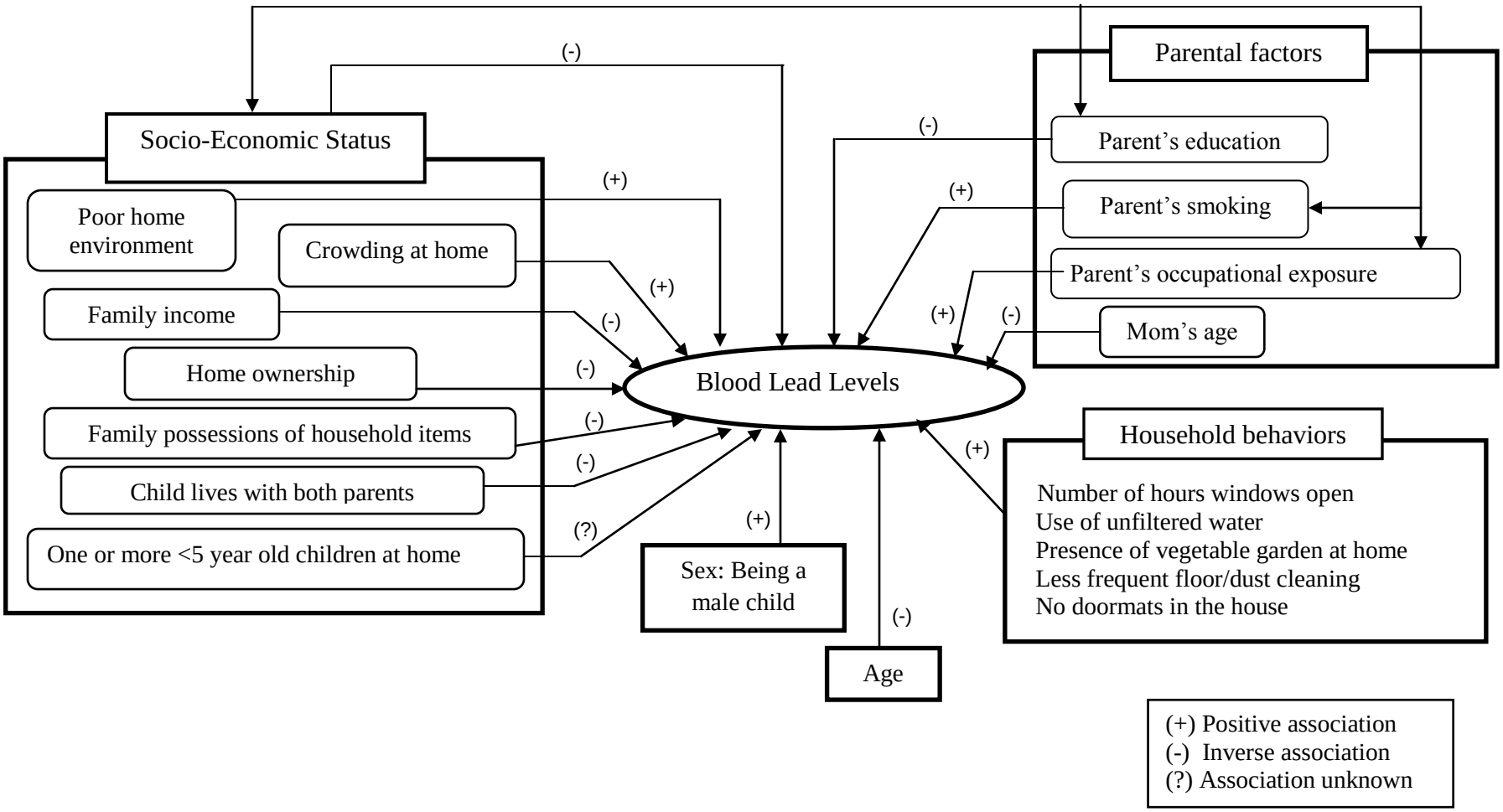

Fig 2: Evidence-based compilation of predictors (socio-demographic and household behaviors) of blood lead levels in school children

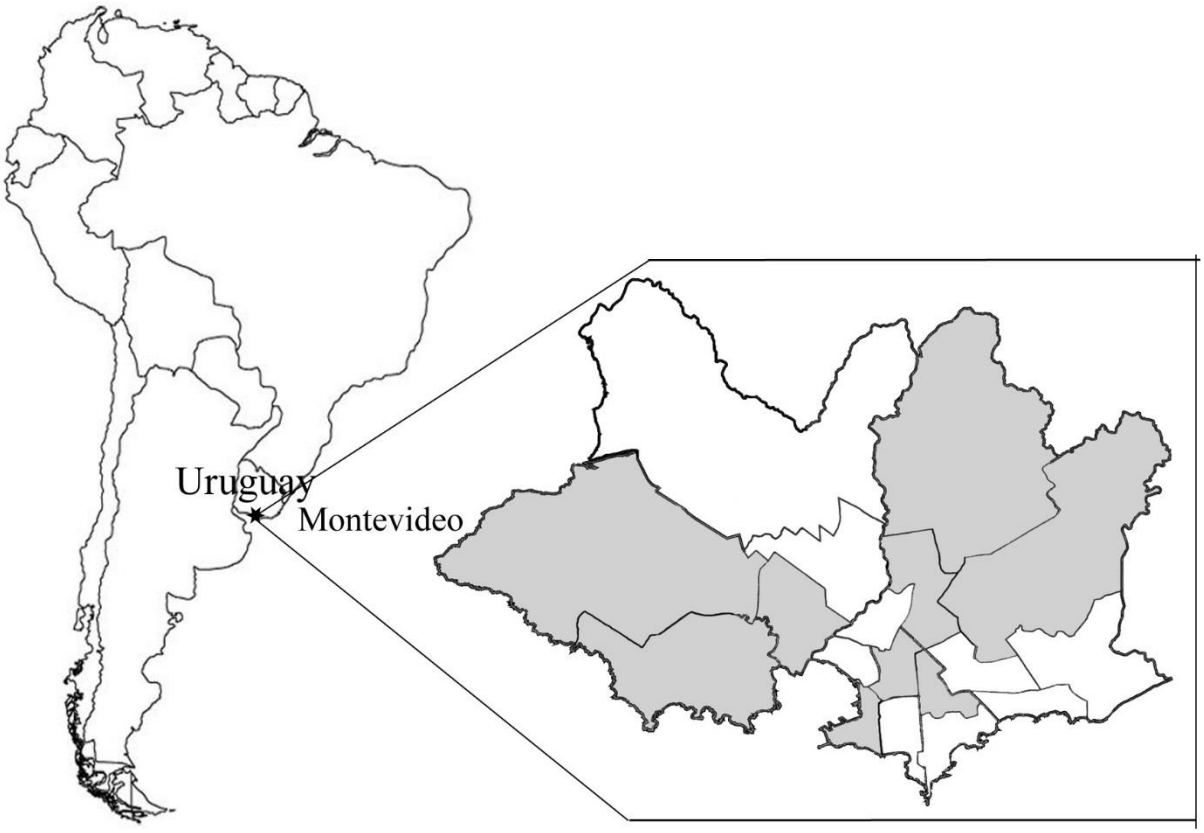

Fig 3: Map of Montevideo with neighborhoods where the study children lived

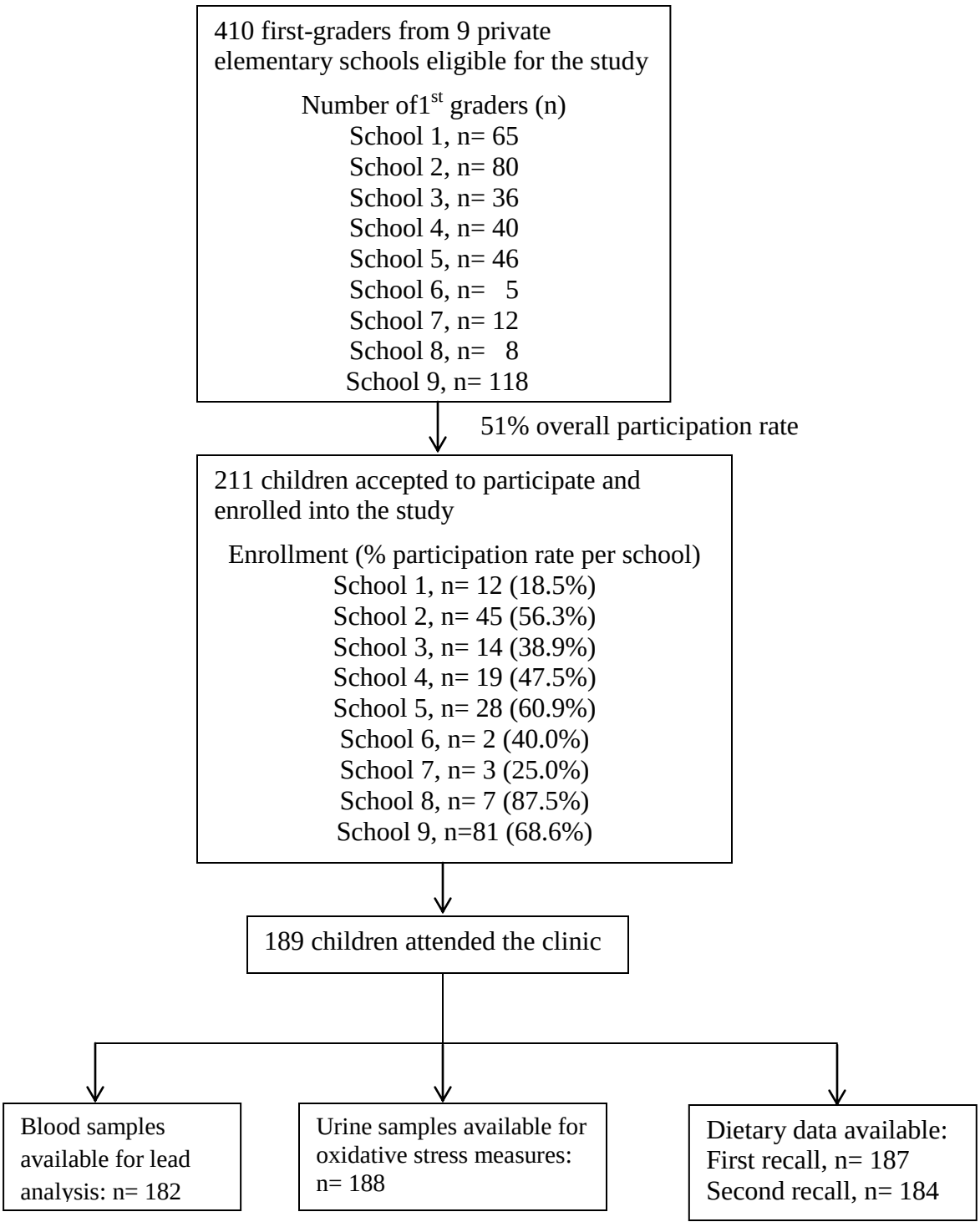

Figure 4: Flow diagram of study participation

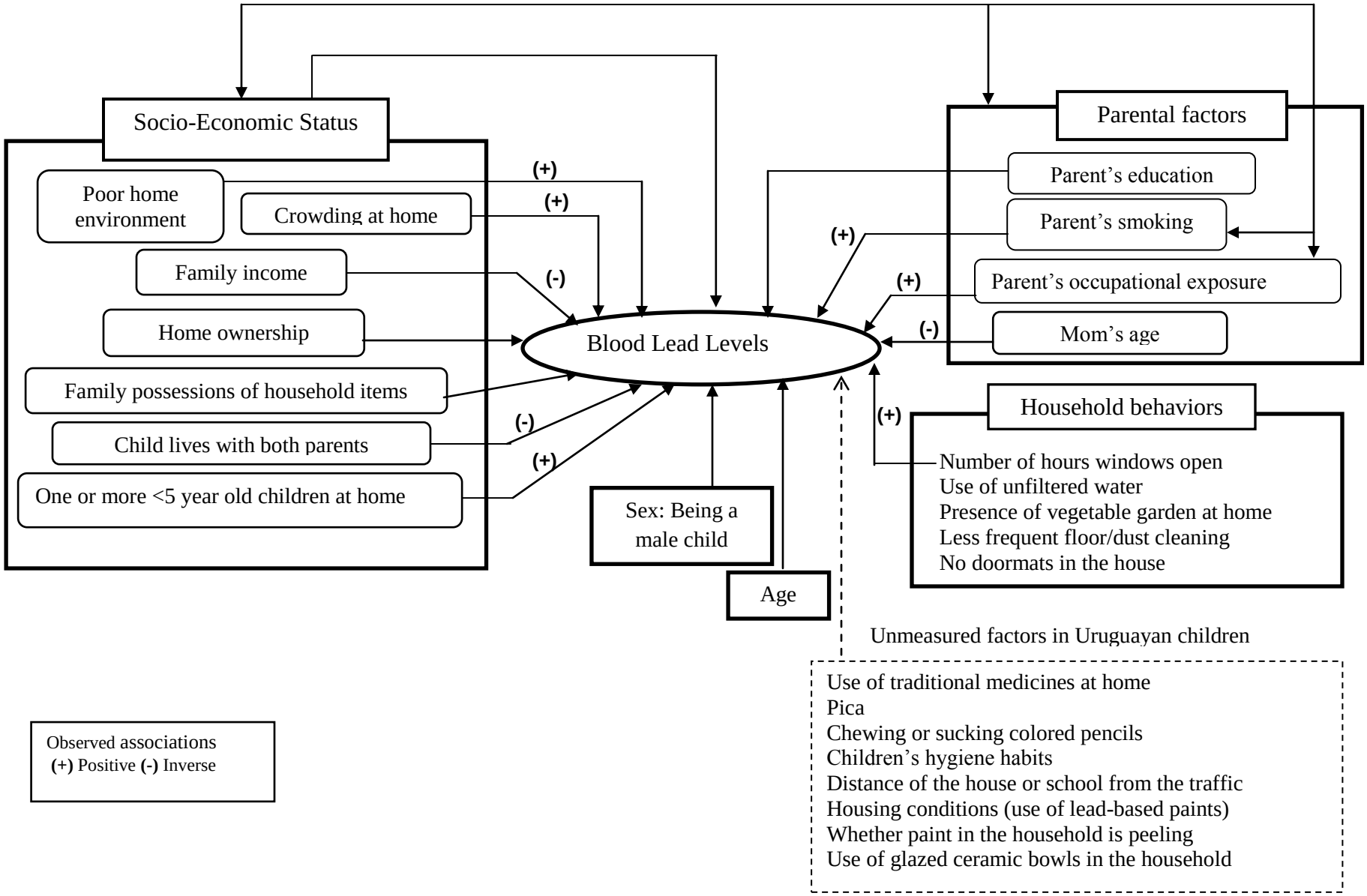

Fig 5: Socio-demographic factors and household behaviors as predictors of blood lead levels in Uruguayan first-graders: observed associations and proposed unmeasured factors

Chapter 4

Micronutrient intake and blood lead levels in Uruguayan school children

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ABSTRACT:

There is some evidence that nutrient intake/status of young children and adults may modulate lead exposure. Yet, the relationship between dietary intake of nutrients and blood lead level (BLL) is not well characterized. The objectives of this cross-sectional study were to examine the relation between BLL and dietary intakes of total energy, carbohydrate, protein, fat and micronutrients (calcium, iron, zinc, vitamin C and folate) and to explore interactive effects of iron-zinc, iron-vitamin C and iron-calcium (which may potentially affect absorption of these micronutrients) on BLL in 211 children (5-8 year olds) from Montevideo, Uruguay. Dietary nutrient intake was assessed by two 24-hour diet recalls. Children had a mean BLL of 4.7 (SD 2.2) μg/dL, with 30% children having a BLL≥ 5 μg/dL (current reference level set by the Centers for Disease Control). In covariate-adjusted multiple regression analyses, there were positive associations between dietary carbohydrate intake, percentage of energy intake from fat (fat %) and BLL; a dose-response relationship was not established between tertiles of carbohydrate or fat % and BLL. Calcium intake was inversely associated with BLL in a dose-related manner. Children in the highest tertile of calcium intake had lower BLL than those in the lowest tertile of calcium [β= -0.8; $p< 0.1$]. There was a 76% lower likelihood of having BLL≥ 4.9 μg/dL (median BLL) among children in the highest tertile compared to the lowest tertile of calcium intake ($p< 0.05$). No association was found between the intake of other nutrients of interest and BLL. There were no interactions between the pairs of micronutrients. Prospective studies and well-designed interventions are required to confirm our findings and to establish effective dietary strategy against lead exposure.

Keywords: lead, children, nutrition, macronutrient, micronutrient

1. INTRODUCTION:

Childhood lead exposure, especially at low levels, continues to be a significant environmental health concern around the world (Attina &Trasande, 2013). The idea that nutrition may modulate the vulnerability to lead exposure and its insults is not new (Mahaffey, 1986). Yet, the nature of this relationship and/or the extent to which nutritional factors can modify exposure or toxicity is not fully elucidated. This understanding is important to establish a dietary strategy or recommendation to minimize the risk of exposure and the toxic effects of lead.

Deficiencies or excess of nutrients can alter the levels of lead in the body. For example, studies have shown that iron deficient children had higher blood lead concentration (BLL) than iron-replete children (Kwong et al., 2004; Wright et al., 2003; Bradman et al., 2001; Wright et al., 1999). An inverse association between iron intake and BLL has also been reported in some studies (Hammad et al., 1996; Lanphear et al., 2002; Lucas et al., 1996; Schell et al., 2004). Similarly, higher intake of calcium has been associated with lower BLL in children (Elias et al., 2007; Lacasaña et al., 2000, Mahaffey et al., 1986; Schell et al., 2004). An inverse association between zinc intake and BLL has been observed in experiments with animals (Cerklewski, 1984; Cerklewski & Forbes, 1976; Jamieson et al., 2006) and in a study of children (Schell et al., 2004). High levels of serum vitamin C were associated with a lower prevalence of elevated BLL ($\geq$ 15 μg/dL) (Simon and Hudes, 1999).

In contrast, the relation between total calorie or macronutrient intake with BLL is mostly unknown. Higher calorie and fat intake has been associated with higher BLLs in some studies (Gallichio et al., 2002; Lucas et al., 1996). Results related to protein intake are mixed, with some showing positive association between dietary protein and BLL in infants (Penuela et al., 2006; Schell et al., 2004) and others observing inverse or no associations (Lucas et al., 1996; Mooty et

al., 1975; Quarterman et al., 1978). Nevertheless, most of the studies examining nutrient intake and lead exposure, looked at the relationship between a single nutrient and biomarker of lead. However, instead of a single nutrient, it is important to evaluate the relationship between lead and nutrients in light of the overall diet because multiple nutrient deficiencies/excess is common and because the nutrients interact with each other inside the body which can affect the finding.

The interplay between nutrients and lead is a complex process and not fully understood (Kordas et al., 2007). One of the ways lead and nutrients interact is possibly through the gastrointestinal absorption. Ingestion of lead is the primary route for exposure in children and ingested lead enters the body through the intestine, as do the nutrients. It is thought that lead competes for common transporters used for the absorption and transport of iron (DMT1) (Bannon et al., 2002, 2003), calcium (calbindin-D) (Fullmer, 1992) and zinc (ZIP-4, cysteine-rich intestinal protein or CRIP) (Jamieson et al., 2007). Other factors, such as bile acid, which helps in the digestion and absorption of fat, may also increase lead absorption (Bell & Spickett, 1983; Hillburn et al., 1980). So far, much of the biological mechanism behind lead absorption in the body remains unknown. It is possible that lead is an opportunistic metal and uses multiple transporters or routes for absorption.

Several studies have examined the efficacy of supplementation of micronutrients such as calcium (Canfield et al., 2005; Keating et al., 2011; Markowitz et al., 2004; Sargent et al., 1999), iron (Ruff et al., 1993; Wolf et al., 2003; Zimmermann et al., 2006), and iron & zinc (Rosado et al., 2006) with limited success in lowering children's BLL (Kwong et al., 2004). Additional interventions are warranted to identify the groups of children who will most benefit from the supplementation of nutrients. However, before conducting such expensive trials, it is important to characterize the relation between nutritional status/intake and BLL. Studies examining the

dietary intakes and BLL are limited (Elias et al., 2007; Hammad et al., 1996; Lacasaña et al., 2000; Lanphear et al., 2002; Lucas et al., 1996; Mahaffey et al., 1986; Mooty et al., 1975; Penuela et al., 2006; Quarterman et al., 1978; Schell et al., 2004; Simon and Hudes, 1999) and very few have looked at the effects of interactions between the micronutrients on modifying lead exposure in children (Rosado et al., 2006). Many nutrients interact and affect each other's absorption (Sandstrom, 2001). This, in turn, may have implications for the intestinal absorption of lead.

The primary objective of the current study was to investigate the association of dietary intakes of total energy, carbohydrate, protein, fat and micronutrients (iron, zinc, calcium, folate and vitamin C) with BLL in 5-8 years old Uruguayan children. An additional aim was to examine the potential effects of interactions among pairs of nutrients (iron-vitamin C, calcium-iron and iron-zinc) on BLL. Our decision to investigate these nutrients was based on previous literature showing potential interactions with lead and the availability of information on the nutrient composition of Uruguayan food items.

2. METHODS:

2.1. Study setting and participant recruitment:

This study was conducted in private elementary schools located in nine municipal areas (Centro Communal Zonal) in Montevideo, Uruguay. The study was approved by the ethics committees at the participating institutions, the Pennsylvania State University, the Catholic University of Uruguay, and the University of the Republic of Uruguay. The study setting and participant recruitment have been described elsewhere (Chapter 3 of the dissertation). Of the 410 eligible children, 211 agreed to participate and enrolled into the study.

2.2. Procedure:

Children were invited to attend a morning evaluation session to collect fasting biological samples, anthropometric measurements, and 24-hour dietary recalls. Children were accompanied by their parents or caregivers. At the end of the clinic, children received a light breakfast. On another day, parents or caregivers filled out a demographic questionnaire in a meeting held at the school. Parents were compensated for their participation with a merchandise voucher.

2.2.1. Parental questionnaire:

Parents/caregivers filled out a questionnaire on socio-demographic characteristics of the family, the child's medical history and the home environment. A detail description of the parental questionnaire has been provided elsewhere (see chapter 3). The questionnaires were self-administered but the research staff provided assistance if parents or caregivers asked for clarification.

2.2.2.. Anthropometric measurements:

Children's height was measured in triplicate to the nearest of 0.1 cm, using a portable stadiometer (Seca 214, Shorr Productions, Colombia, MD). Children were weighed in triplicate to the nearest 0.1 kg using a digital scale (Seca 872, Shorr Productions, Colombia, MD). Both measurements were carried out by a trained pediatric nurse or nutritionist. Children were asked to remove their sweaters or jackets but did retain light clothing like school uniforms. The three measurements were averaged, and a final weight was calculated by subtracting standard weights of children's clothing, taking into account the clothes each child wore at the time of measurement. The following standard clothing weights were applied to the body weight measurements: sweatshirt (255 g), T-shirt (85 g), polo shirt (110 g), sweatpants (155 g), jeans (340 g). Based on the mean weight (corrected for clothing) and height measurements, the child's

BMI was calculated. Z-scores on weight-for-age, height-for-age and BMI-for-age were calculated using the WHO Anthro software (WHO, 2010). A BMI-for-age Z score >+1 SD and >+2 SD were used as the cut-offs for overweight and obesity, respectively (WHO reference 2007).

2.2.3. Blood collection and processing:

Fasting venous blood sample was collected by a phlebotomy nurse. Approximately 3 ml of blood was collected from each child using a 25-gauge safety butterfly blood collection set (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) in heparin coated trace-metal free tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for lead analysis. An additional 3 ml of venous blood was drawn in a serum tube with clot activator and separator gel (Becton Dickinson, Franklin Lakes, NJ).

Serum was separated from blood collected in serum tubes by centrifuging the samples for 10 min at 3000 rpm. Approximately 250 µl of serum was aliquoted for serum ferritin (SF) and C-reactive protein (CRP) measurements. The whole blood and serum samples were stored on ice until their transport to the Toxicology Laboratory (CEQUIMTOX) in the Faculty of Chemistry at the University of the Republic, Montevideo, Uruguay, where whole blood tubes were stored at -20°C until analysis. Serum was stored at -20°C at the Research Center, Catholic University of Uruguay (Montevideo, Uruguay) until their subsequent shipment to the Pennsylvania State University for analysis.

2.2.4. Blood lead, haemoglobin, serum ferritin and CRP analyses:

Blood lead analyses were performed at the Laboratory CEQUIMTOX (Specialized Center for Chemical Toxicology), the Faculty of Chemistry, University of the Republic, using Atomic Absorption Spectrometry (AAS, VARIAN SpectrAA-55B) with flame or graphite

furnace ionization techniques, depending on the volume of whole blood available. The detection limit was 1.8 µg/dL for the flame and 0.8 µg/dL for graphite furnace AAS techniques. Analytical conditions were validated with standard quality assurance/ quality control (QA/QC) procedures (Parson and Chisolm, 1997). The laboratory participates in the Inter-laboratory Program for Quality Control for Lead in Blood, Spain and the US Centers for Disease Control's Lead and Multi-Element Proficiency Program.

A drop of venous blood was immediately removed from the serum tube and hemoglobin was measured using a portable hemoglobinometer (HemoCue Inc, Lake Forest, CA), which was calibrated daily using standard HemoCue controls provided by the manufacturer.

Serum ferritin concentrations were determined in duplicate by an immunoradiometric assay kit (Coat-A-Count Ferritin IRMA; SIEMENS Diagnostic Products, USA). The samples were allowed to come to room temperature and mixed gently by swirling before use in the assay. An aliquot of 10 µl of serum was used for the analysis. The Coat-A-Count IRMA is a solid-phase immunoradiometric assay based on one ^{125}I- labeled anti-ferritin polyclonal antibody in liquid-phase and a monoclonal anti-ferrtin antibody coated to the wall of a polysterene tube. Ferritin is captured between these two antibodies. The radioactivity of the ^{125}I-labeled poly clonal anti-ferritin antibody (tracer) was measured using a gamma counter (Packard Cobra II Auto gamma counter, Perkin Elmer). The ferritin concentration is directly proportional to the radioactivity (measured as count per minute) present in the tube. The concentration of serum ferritin was calculated by using an eight point standard curve obtained from the set of calibrators provided by the manufacturer. Intra-assay and inter-assay coefficients (CV) were calculated to determine the variability within and between the assays, and were 4.2% & 9.5% respectively.

Concentration of CRP was measured to identify the presence of subclinical inflammation/infection in the study children. CRP was analyzed in duplicate using an ELISA technique described by Erherdt and colleagues (2004). Goat anti-Human CRP antibody (Bethyl Laboratories, TX) and HRP conjugated Goat anti-human CRP antibody (Bethyl Laboratories, TX) were used, respectively, as capture and detection antibodies. Serum control samples (Liquicheck, Bio-Rad) were used as standards. The plates were read at 450 nm by an automated micro plate reader (Epoch Microplate Spectrophotometer, Biotek, Winooski, VT). The serum levels of CRP were determined from a seven-point standard curve. Intra-assay and inter-assay coefficients (CV) were 4.9% and 8% respectively.

Serum ferritin concentrations were adjusted for the presence of inflammation by adjusting for serum CRP concentration using a method described by Thurnham and colleagues (2010). First, children were stratified as having inflammation (CRP> 5 mg/L) or no-inflammation (CRP≤ 5 mg/L) according to their CRP levels. Then, individual ferritin values of the children with inflammation were adjusted by multiplying by the ratio of the geometric mean of serum ferritin in the inflammation group to the geometric mean of serum ferritin in the non-inflammation group (0.63 for this study).

2.2.5. Assessment of nutrient intake:

Information on dietary intake of the participating children was assessed using two 24-hour diet recalls- one recall took place at the school on the day of the blood draw and the second recall took place over the phone without prior appointment, at least 2 weeks later, either on a weekday or a weekend. All the recalls were conducted by five trained nutritionists with the mother or another caregiver familiar with the child's diet. The child was present at the time and contributed to the recall, particularly with the recollection of food consumed at school. Three

separate contact attempts were made by telephone to complete the phone interview. The recall was not conducted if all attempts went unanswered or the primary caregiver was not available.

A detailed list of all the foods and beverages the child consumed within the previous 24-hour period was collected. Information was obtained about the name of the meals, time and place of consumption, amounts of foods consumed or food portions, food preparation methods, recipe ingredients and brand names of commercial products. Food models and household measurement cups were used during the interview with the mothers or caregivers to facilitate food portion recalls, and to quantify the amount and volume of foods/beverages consumed (Compendio de Referencias Prácticas, Oficina del Libro FEFMUR, Montevideo, 2002; Vázquez and Witriw, 1997). Use of vitamin and mineral supplements, not very common in this population, was also queried. Neutral probing questions such as "Did your child eat/drink anything on the way home from school yesterday?" "Did he/she have anything before going to bed?" were asked to get accurate dietary information. All the foods were assigned a unique code and entered, along with amounts consumed, into a database that contained the nutrient composition of typical Uruguayan foods and preparations (altogether 342 unique items). The current mineral fortification laws in Uruguay were taken into consideration in the database. Since 2006, all commercially produced wheat flours in Uruguay are fortified with 2.4 mg of folic acid and 30 mg of elemental iron per kg. Milk distributed in school-based lunch programs is also fortified with iron under the same law. Calculations of nutrient intakes were derived from the database.

2.3. Statistical analysis:

Data were analyzed using STATA 12.0 (STATA Corp, College Station, TX).

2.3.1. Covariates selection:

The significant socio-demographic predictors of BLL of the study children were identified previously (chapter 3) and were used in the final regression analyses as the covariates in the current study. Briefly, a two-step approach was adapted to detect the salient socio-demographic and household behavioral factors predictive of children's BLL. First, all child and family characteristics along with household factors were tested individually in bivariate regression model. Next, the variables which were associated with BLL at $p< 0.25$ in bivariate analysis were entered into a multiple regression model, and variables with $p< 0.1$ were retained in the multivariate model with the help of a backward stepwise variable deletion process. The socio-demographic predictors identified by the above method were father's occupation with potential for lead-exposure, having more than one young sibling in the household and father's smoking. Covariates were also chosen based on previous literature or if they were associated with the nutrients in bivariate regression analyses (p-value< 0.1). Thus CRP-adjusted serum ferritin was a significant predictor of children's BLL ($p< 0.05$) and was adjusted for in the regression models, along with children's BMI.

2.3.2. Dietary data analysis:

Dietary nutrient intake data from two 24-hour recalls were averaged for each individual nutrient. In addition, proportion of energy from carbohydrate, protein and fat was also calculated. All the nutrient intake values were adjusted for total energy intake using the nutrient residual model approach (Willett 1998). Calorie-adjusted nutrient intakes were computed from the mean of unadjusted nutrient intakes plus the residuals from the regression model with total calorie intake as the independent variable and each nutrient intake as the dependent variable (Willett 1998). The rationale behind energy adjustment is that individual differences in total energy

intake produce variation in intake of specific nutrients unrelated to dietary composition, and it has been shown that the consumption of most nutrients is positively correlated with total energy intake (Willet et al., 1997).

2.3.3. Complete-case analysis:

A series of multiple linear and logistic regression analyses was performed to examine the independent association between the intake of total energy, carbohydrate, protein, fat, the micronutrients- iron, zinc, calcium, folate, vitamin C, and children's BLL. BLL was entered as a dependent continuous or categorical (dichotomized at median BLL- 4.9 µg/dL) and nutrient intakes as independent variables categorized as tertiles. Each nutrient was tested in a separate model (Models 1-4 for total energy, carbohydrate, protein, and fat; Models 8-12 for iron, zinc, calcium, folate, and vitamin C). The association of BLL with percentage of energy-contribution from carbohydrate, protein and fat was also tested in separate multivariate models (Models 5-7). All regression models were adjusted for a core set of covariates described above.

The potential interactive effects between pairs of nutrients (iron-vitamin C, calcium-iron and iron-zinc) were tested in covariate-adjusted multiple linear and logistic regression models (Models 13-15). All the interactions were tested in separate models. Interaction terms were created by crossing each tertile of a nutrient with corresponding tertile of another nutrient. For example, the interaction term for iron-vitamin C was constructed by crossing each tertile of iron with corresponding tertile of vitamin C intake. Thus three interaction terms were used for iron-vitamin C, with interactions between the lowest tertiles of iron and vitamin C intakes being the reference level in the regression models. Interactions between other pairs of micronutrients (calcium-iron and iron-zinc) were created in a similar manner. Non-significant interactions (p>

0.15) were removed and models were re-run testing the main effects of calcium and iron (Model 13), iron and zinc (Model 14), and iron and vitamin C (Model 15).

2.3.4. Sensitivity analysis:

The 95th percentile for calorie intake among the study children was 3955 Kcal/day. We repeated the analyses excluding children with calorie intake of more than 3955 Kcal (n= 2).

2.3.5. Missing data analysis:

In the complete-case-analysis, children's BLL, nutrient intakes and all the possible predictor variables except for sex and school were subject to missing data. Muiltivariate multiple imputation was used to impute missing variables. The imputation model included BLL, other biomarkers such as hemoglobin and serum ferritin concentrations, and the socio-demographic variables from 211 children who completed the study. Interaction terms between two micronutrients (as tertiles) were also included in the imputation model. Imputation by chained equations (command "mi ice") of STATA version 12.0 (StataCorp, College Station, TX) was conducted (Royston and White, 2011). Briefly, the algorithm of "mi ice" works in the following manner: first, each variable with missing values is regressed on the other variables in the imputation model. Next, missing values in each variable are replaced by simulated draws from the posterior predictive distribution of the variable derived from the regression estimation. The process is repeated for all the incomplete variables in the imputation model and the entire round is called a cycle. To stabilize the results, the regression switching cycle is iterated 10 times (default in STATA) and finally a single imputed dataset is generated. In total, 10 imputed datasets were created for the analyses.

3. RESULTS:

3.1. Characteristics of study children:

In total, 182 children provided blood for lead analysis and information on daily intake of nutrients was available for 187 children (Fig 3, chapter 3). The mean (standard deviation or SD) age of the study children was 6.8 (0.6) years (**Table 15**). Children in this study had a mean BLL (SD) of 4.7 (2.2) µg/dL, with 30.2% children having a BLL≥ 5 µg/dL, current reference level set by US Centers for Disease Control (CDC 2012) for identification, monitoring and management of children with elevated BLL. Only 3% of the study children were anemic (Hb< 11.5 g/dL), but more than half (61.2%) were iron-deficient. The mean (SD) CRP-adjusted serum ferritin level was 14.3 (13.2) ng/ml. The mean (SD) body mass index (BMI) was 16.9 kg/m^2, with 19.8 % of the children each being overweight (BMI-z-score> +1 SD) and obese (BMI-z-score> +2 SD) according to WHO standards (WHO reference 2007). Fathers of 51.9% children were involved in jobs with potential for metal exposure such as construction, factory work, print shop, mechanic or driver. While 41.8% fathers reported to be currently smoking, mothers of 35.6% children were smokers. About 22.6% of the children lived in a crowded (>2 people/room) household. Mean number of "luxury" items present at children's homes was 3.6 (out of six items), with 42.6% families having equal to or less than three items. More than half of the households had at least one young child (< 5y) at home.

3.2. Children's dietary intake:

The mean (SD) daily calorie intake of the study children was 2253 (603) Kcal/day (**Table 16**). Boys had higher calorie intake than girls [2369 (SD 588) Kcal vs 2086 (SD 588) Kcal; p< 0.05]. Total protein and fat intake also differed between the sexes, with boys having higher intakes of the macronutrients than the girls. The mean (SD) percentage of energy from

carbohydrate, protein and fat in the entire sample was 56.1 (7.4), 13.8 (7.3) and 30.9 (6.5)%, respectively. There were no sex differences in the percentage of energy contribution from any of the macronutrients. The mean (SD) calcium intake of the study children was 738 (282) mg/day. More than half of the children (62.6%) did not meet the Estimated Average Requirements (EAR) for calcium (800 mg/day) (IOM, USA) and 40.7% children did not meet the Recommended Dietary Allowance (RDA) established for Uruguayan children (700 mg/day). Majority of the children met the EAR (4.1 mg/day) and RDA (7 mg/day) for iron intake. There were sex differences in mean intake of zinc, with girls having lower zinc intake than the boys [4.7 (SD 2.3) vs 5.6 (SD 2.5) mg/day; $p< 0.05$]. About 17.6% and 37.4% children did not meet the EAR (25 mg/day) and RDA (35 mg/day) for vitamin C established for Uruguayan children. Finally, while majority of the children met the EAR (160 µg/day) for folate intake, only 15.4% children did not meet the RDA (330 µg/day) for Uruguayan children.

3.3. Complete-case analysis:

The association between intakes of total calorie, the macronutrients (carbohydrate, protein, fat) and children's BLL was examined in separate covariate-adjusted regression models where dietary intakes were entered as tertiles and children's BLL as continuous or categorical variable (categorized at median BLL: 4.9 µg/dL) (Models 1-4, **Table 17**). For the multivariate complete-case analyses, data were available for 111 children. No significant associations were observed between total calorie, protein or fat intake and BLL. Compared to the children in the lowest tertile of carbohydrate intake, those in the second tertile had higher BLL [$\beta= 1.22$; $p< 0.05$] and higher likelihood of having BLL$\geq$ 4.9 µg/dL [OR= 3.04; $p< 0.05$] (Model 2, Table 17). When the association between energy percentages from carbohydrate, protein, fat and BLL was tested in multivariate regression models (Models 5-7, Table 17), children in the second tertile of

percent energy as carbohydrate had higher BLL [β= 1.32; p< 0.05] and higher likelihood of BLL≥ 4.9 µg/dL [OR= 4.79; p< 0.05] than those in the lowest tertile. Similarly, compared to the lowest tertile of percent energy as fat, children in the second tertile had higher BLL [β= 1.02; p< 0.05] (Table 17). However, no difference in BLL was found between those in the first and third or second and third tertiles of these nutrient intakes.

The relation between micronutrient intake (calcium, iron, zinc, vitamin C and folate) and children's BLL was tested in separate multiple regression models (Models 8-12, **Table 18**). In covariate-adjusted multiple linear regression analysis, compared to the children in the lowest tertile of calcium intake, children in the highest tertile had 0.8 µg/dL lower BLL (p< 0.1) (Model 8, Table 18). In multiple logistic regression models, children in the highest tertile of calcium intake had 76% lower risk of having BLL≥4.9 µg/dL than children in the lowest tertile of calcium (p< 0.05) (Model 8, Table 18). Intakes of other nutrients, such as iron, zinc, folate and vitamin C were not associated with BLL or with the likelihood of having BLL≥ 4.9 µg/dL (Models 9-12, Table 18).

There were no statistical interactions (p> 0.15) between the pair of nutrients: iron-calcium, iron-vitamin C and iron-zinc (Models 13-15, Table 18). Next, the interaction terms were removed and analyses were repeated to test the main effects of iron and calcium (Model 13 Table 18), iron and vitamin C (Model 14, Table 18), and iron and zinc (Model 15, Table 18). Calcium intake continued to be inversely associated with BLL in a dose-related manner when entered together with iron (Model 16, Table 18). No other significant main effects of micronutrients were observed.

3.4. Sensitivity analysis:

When we repeated the analyses excluding children with calorie intake more than 3955 Kcal (n= 2), the results did not significantly differ from the complete-case analyses with those children.

3.5. Missing data analysis:

When the analyses were rerun with multiply imputed datasets, the results were similar to those found in the complete-case analysis (**Table 19 & 20**).

4. DISCUSSION:

In this cross-sectional study of 211 first-grade children, we found positive associations between BLL and the dietary intake of carbohydrate (both in absolute terms and as a percentage of total energy), as well as percentage of energy from fat. However, we did not find a clear dose-response relationship between BLL and these two macronutrients. Specifically, children in the second tertile of dietary carbohydrate (absolute value or percent energy) and fat percent had higher BLL than those in the first or third tertile. We failed to detect differences in BLL between first and third or second and third tertiles, possibly due to a narrow range of BLLs in the study sample. In addition, the difference in median energy intake between second and third tertile did not differ significantly, thus providing less statistical power to detect differences among the other groups.

Among the micronutrients, higher dietary intake of calcium was associated, in a dose-related manner, with lower BLL in the study children and this result remained consistent in models that examined calcium and other nutrients concomitantly. Children in the highest tertile of calcium intake (>850 mg/day) had lower BLL than children in the lowest tertile of calcium

(<626 mg/day). The likelihood of having higher than median BLL (≥ 4.9 μg/dL) was also lower among children in the highest tertile compared to those in the lowest tertile of dietary calcium.

Only a handful of studies had assessed the relation between dietary intakes of both macro and micronutrients, and children's BLL (Elias et al., 2007; Gallicchio et al., 2002; Lucas et al., 1996; Schell et al., 2004;). Elias and colleagues (2007) conducted a study in 6-9 year olds to examine the relationship between dietary intake of nutrients and BLL. The authors modeled the intakes of macro and micronutrients and the socio-demographic factors as independent variables in a multivariate regression model and used the stepwise method to choose the most salient predictors of children' BLL. In that model, none of the nutrients was significantly associated with BLL. The authors attributed the null findings to low BLLs in their study children. Because a large number of their study children did not have adequate energy intake, when the authors restricted their analysis to children who had inadequate energy intake, high dietary protein was inversely associated with BLL. Similarly, dietary calcium intake was inversely related to BLL in children who did not meet the recommended intake (Elias et al., 2007). Schell and colleagues (2004) found inverse relationships between dietary protein, iron, zinc and calcium and BLL of 6-month-old infants, and an inverse relationship between dietary iron and BLL when those children were again assessed at 12 months. Positive relationships were observed between the intakes of total fat, saturated fat and BLL in 1-year old children (Gallicchio et al., 2002). Finally, in a study with 9-72 month old children, total energy and fat intake were positively associated with children's BLL (Lucas et al., 1996).

We observed an inverse relation between dietary protein intake and children's BLL in this study, but it did not reach statistical significance. The evidence concerning the link between dietary protein and children's BLL is inconsistent, with some studies showing a positive

(Penuela et al., 2006; Schell et al., 2004) and others showing null (Lucas et al., 1996) or inverse relationship (Mooty et al., 1975; Quarterman et al., 1978). On the other hand, tofu (has a high protein content) intake was inversely associated with BLL in Chinese adults (Chen et al., 2001). Because tofu also contains high amounts of calcium, it is not clear whether the protective role of tofu against BLL was due to its protein or calcium content. The biological link between protein and lead is unknown, but it is suggested that the amount of protein intake may affect lead absorption (Barltop & Khoo, 1975). We did not observe any association between children's total energy intake and BLL. The mean daily calorie intake of the study children was quite high (2253 kcal/day) and comparable to that of US children (Ervin & Ogden, 2013). There is some suggestion that food consumption is normally high in individuals with high calorie intake and food contaminated with lead can be a source of exposure (Lucas et al 1996). In contrast, low calorie intake may represent low food consumption and lead absorption may also increase if the stomach is empty (Quarterman et al., 1978; Rabinowitz et al., 1980). However, all macronutrients contribute to total calorie intake, so it is difficult to discern whether it is the effect of nutrients or the total food consumption that is being assessed when the relation between total calorie and BLL is examined.

Ours is one of the few studies to report an association between carbohydrate intake and BLL, previous studies did not find any significant association (Elias et al., 2007; Lucas et al., 1996), except a study in infants that reported an unadjusted inverse relation between the intake of carbohydrate and BLLs (Gallichio et al., 2002). The biological connection between carbohydrate and lead is not known, it is possible that children with higher carbohydrate intake had more food consumption and food itself could be a source of lead exposure. Previous studies reported an inverse relation between fat intake and BLL (Gallichio et al., 2002; Lucas et al., 1996). Although

we did not find any significant relation between total fat intake (absolute value) and children's BLL; fat intake expressed as a percentage of total daily energy intake was positively associated with BLL. Because all the macronutrients (carbohydrate, protein, fat) contribute to dietary energy intake and because the energy intake widely varies between individuals, the proportions rather than the absolute values of macronutrients may actually reflect the usual intake of a nutrient. In addition, for a given energy intake, increases in the proportion of one macronutrient involve a decrease in the proportion of one, or more, of the other macronutrients, which may have important implications for children's health including BLLs.

On the other hand, the observed association of higher calcium intake with lower BLL is consistent with previous studies in children (Lacasaña et al., 2000; Mahaffey et al., 1986; Schell et al., 2004). However, Lanphear and colleagues (2002) did not find any significant association between calcium intake and BLL when they followed a group of infants from 6 to 24 months. Notably, children in their study had a very high calcium intake at every stage of infancy. In addition, the age group at which children were being studied is important since younger children normally have higher BLL (Dietrich et al., 2001) and their dietary patterns also differ considerably from older children. The diets of infants and pre-school children normally consist of fairly high amounts of dairy products. This makes the result of our study of particular interest since the study children were older, had low-level lead exposure (commonly observed in general population) and lower calcium intake than children in previous studies.

We failed to observe any significant associations between the intakes of iron, zinc, vitamin C or folate, and BLL in these children. The interactions between pairs of micronutrients (iron-calcium, iron-vitamin C or iron-zinc) did not modify the relation between micronutrients and BLL in our study children. Some studies found inverse associations between dietary iron

(Hammad et al., 1996; Lanphear et al., 2002; Schell et al., 2004;), zinc (Hammad et al 1996; Hertz et al 1998; Schell et al 2004) and vitamin C (Schwartz et al., 1989), and BLL in young children. Studies with folate intake and BLL are rare. To our knowledge, ours is one of the first studies examining the relation between folate intake and BLL in children. There may be several reasons behind the null associations in our study. First, intakes of these nutrients were adequate in the majority of study children. With adequate intake, there may not be an additional benefit of these micronutrients on children's BLL. Second, unlike previous studies, children in this study were older. It is known that during the first year of life, infants are vulnerable to low iron intake if adequate iron is not provided by the complementary foods since breast milk provides a small amount of bioavailable iron (Duggan et al., 1998). But as children grow older, more foods are introduced in the diet and the possibility of getting adequate iron from the diet increases. Although many children in our study had low ferritin levels. In the presence of adequate dietary iron intake, we could only speculate that ferritin levels were low due to insufficient iron absorption as a result of inadequate vitamin C intake or inhibitory effects of other dietary factors. Presence of gastrointestinal (GI) disorders (such as celiac or Crohn's disease, GI bleeding) or parasitic infection (for example *H. Pylori*) can also cause low serum ferritin. Our data suggests that vitamin C intake in majority of the study children was adequate. Confirmation of vitamin C status is only possible with data on plasma levels of vitamin C of these children. We don't have information on plasma vitamin C or dietary factors that may inhibit iron absorption in these children. Furthermore, we tested CRP levels to detect the presence of infection or inflammation, and only 10% children had elevated CRP concentration, suggesting that inflammation or infection does not play a significant role in determining iron absorption or status in these children.

Some limitations need to be considered while interpreting the findings of this study. First, this was a cross-sectional study, thus no cause-effect relationship can be established nor can it delve into the biological mechanism behind the observed findings. Second, the quality of dietary data depends on the collection method and the recall capabilities of the respondents- to address this, recalls were facilitated by probing about specific meals in detail, and by using food models and household measurement cups to facilitate food portion recalls, and to quantify the amount and volume of foods/beverages consumed. The accuracy of the dietary data also depends on the nutrient database used to calculated nutrient values. The nutrient database for this study is widely used in Uruguay and it had nutritional information on 342 unique food items, thus capturing an extensive range of Uruguayan foods. Another limitation of this study is lack of data on biomarkers for most of the micronutrients except for iron. However, because the plasma or serum levels of most micronutrients are under tight physiological regulation, the available biomarkers (such as for zinc and calcium) are not very sensitive or specific to identify deficiencies (Hambridge, 2003; Potischman, 2003). Therefore, information on the intake of these nutrients is important to understand the possible adequacy or deficiencies of these nutrients. Furthermore, our study is limited by a smaller sample size with missing data due to non-responses especially on socio-demographic questionnaires. However, we repeated the analyses in multiply imputed datasets to handle the problem of missing data and our results were consistent with complete-case analyses. Nonetheless, our findings need to be tested in a larger sample in future studies.

Ours is one of the few studies examining the relation between the dietary intakes of nutrients and BLL in school-age children. So far, studies have mostly examined the association between nutritional status (sometimes intakes) of individual nutrients and BLL in infants or

women who are pregnant or breastfeeding. While the information on nutritional status is important, understanding of the relation between nutrient intake and BLL can be crucial especially for formulating dietary recommendation because it is thought that lead competes for absorption with specific nutrients. Moreover, extension of findings from other age groups may not be appropriate, since school children are unique in their nutritional needs and deficiencies. As children grow older, requirements of nutrients increase to support their rapid growth and development (Brown, 2008). In addition, with increasing independence and influence from peers, dietary habits of school children may make them susceptible to several micronutrient deficiencies (Brown, 2008). For example, calcium deficiency is prevalent among school children, as milk is replaced by sweetened drinks (Grimm et al., 2004; Nicklas, 2003). Due to low preference for vegetable and fruits, school children are also vulnerable to several vitamin and mineral deficiencies (Krebs-Smith et al., 1996). Furthermore, lead exposure in later childhood contributes to cognitive deficits beyond the effects of early exposures (Canfield et al., 2003). Thus, finding a preventive strategy at this age group is as important as in infancy to reduce BLL and lead toxicity.

Finally, although results from ours and other studies suggest that higher calcium intake may benefit children by reducing their BLL, supplementation with nutrients such as calcium (Keating et al., 2011; Markowitz et al., 2004; Sargent et al., 1999), iron (Wolf et al., 2003; Zimmerman et al., 2006) or iron and zinc (Rosado et al., 2006) has seen limited success in lowering children's BLL. In future, research should focus on conducting prospective studies where children could be followed from infancy to adulthood to better understand the relation between dietary components and to identify the age group that would benefit the most from any dietary intervention.

5. CONCLUSION:

In sum, we found positive associations between dietary carbohydrate and percentage of energy intake from fat, and BLL in 5-8 years old children. No dose-response relationship was found between the intakes of these macronutrients and BLL. Dietary calcium intake was inversely associated with children's BLL. There was no association between the intakes of other nutrients and BLL. No effects of interactions between iron-zinc, iron-vitamin C and iron-calcium was found on BLL. While many of the study children did not meet the recommended intake of calcium, children had adequate intakes for other nutrients. Future research, with larger samples, should focus on conducting a well-designed prospective study to better understand the effects of nutrients and particular components of food on BLL.

Table 15: Participant characteristics

	N	Mean ± SD	%
Child characteristics			
Age (y)	211	6.8 ± 0.6	
Sex	211		
Female			43.1
Blood lead level (μg/dL)	182	4.7 ± 2.2	
≥ 5 μg/dL			30.2
Hemoglobin (g/dL)	184	13.4 ± 1.1	
<11.5 g/dL			3.0
Serum ferritin (ng/mL)[1]	174	14.3 ± 13.2	
<15 ng/mL			61.2
Body mass index (kg/m^2)	187	16.9 ± 2.7	
Overweight[2]			19.8
Obese[2]			19.8
Parental characteristics[3]			
Fathers with job exposure risk[4]	131		51.9
Mother smokes	163		35.6
Father smokes	141		41.8
Household characteristics[3]			
Children <5 y present at home	155		65.2
Crowding (no of person/ no of rooms)	164	2.0 ± 0.8	
Family possessions of household items[5] ≤3 items	184	3.6 ± 1.4	42.6

[1]values adjusted for C-reactive protein levels
[2]calculation based on BMI-z-scores (WHO reference 2007)
[3]based on parental self-report
[4]has exposure risk if employed in jobs such as construction, factory work, print shop, mechanic and driver
[5]items include video-player, computer, car, refrigerator, washing machine and home phone

Table 16: Dietary intakes of nutrients[1]

Nutrients	Mean ± SD			Children not meeting requirement (%)
	Entire sample (n= 183)	Boys (n= 107)	Girls (n= 76)	
Energy (Kcal/d)	2253 ± 603	2369 ± 588	2086 ± 588[2**]	
Carbohydrate (g/day)	314 ± 110	325 ± 112	298 ± 106	
Protein (g/day)	74 ± 32	80 ± 38	66 ± 17[2**]	
Fat (g/day)	77 ± 24	83 ± 25	69 ± 22[2***]	
Energy %				
carbohydrate (%)	56.1 ± 7.4	55.6 ± 6.7	56.7 ± 8.1	
protein (%)	13.8 ± 7.3	14.2 ± 9.1	13.2 ± 3.1	
fat (%)	30.9 ± 6.5	31.5 ± 6.4	30.1 ± 6.6	
Calcium (mg/d)	738 ± 282	764 ± 284	701 ± 277	
US-EAR (800 mg/day)[3]				62.6
UY-RDA (700 mg/day)[4]				40.7
Iron (mg/d)	11.9 ± 8.5	12.1 ± 8.1	11.7 ± 9.1	
US-EAR (4.1 mg/day)[3]				1.1
UY-RDA (7 mg/day)[4]				9.9
Zinc (mg/d)	5.3 ± 2.4	5.6 ± 2.5	4.7 ± 2.3[2**]	
US-EAR (4 mg/day)[3]				34.1
UY-RDA (5.6 mg/day)[4]				59.3
Vitamin C (mg/d)	59.3 ± 53.7	59.9 ± 56.6	58.4 ± 49.6	
US-EAR (35 mg/day)[3]				37.4
UY-RDA (25 mg/day)[4]				17.6
Folate (µg/d)	479.3 ± 187.1	510.8 ± 177.8	434.2 ± 191.8	
US-EAR (160 µg/day)[3]				1.1
UY-RDA (330 µg/day)[4]				15.4

[1]dietary data averaged over two 24-hour diet recalls
[2]intake significantly differs between boys and girls
[3]EAR: estimated average requirement (US IOM)
[4]RDA: recommended dietary intake (Uruguay)
**p< 0.05 ***p< 0.01

Table 17: Association between macronutrient intake and BLL in Uruguayan children[1,2]

Nutrients	BLL (µg/dL)[3] β [95% CI]	BLL≥ 4.9 µg/dL[4] OR [95% CI]
Model 1: energy		
1st tertile (937 – 1961 kcal/d)	Reference	Reference
2nd tertile (1973 – 2447 kcal/d)	0.36 [-0.55, 1.27]	0.81 [0.30, 2.18]
3rd tertile (2465 – 4236 kcal/d)	0.23 [-0.73, 1.19]	1.05 [0.37, 2.96]
Model 2: carbohydrate[5]		
1st tertile (194 – 299 g/d)	Reference	Reference
2nd tertile (300 – 332 g/d)	1.22 [0.31, 2.12]**	3.04 [1.72, 8.62]**
3rd tertile (333 – 437 g/d)	0.53 [-0.38, 1.44]	2.20 [0.79, 6.09]
Model 3: protein[5]		
1st tertile (34 – 64 g/d)	Reference	Reference
2nd tertile (65 – 77 g/d)	-0.49 [-1.40, 0.41]	0.85 [0.32, 2.28]
3rd tertile (78 – 338 g/d)	-0.43 [-1.39, 0.54]	0.63 [0.22, 1.79]
Model 4: fat[5]		
1st tertile (19 – 70 g/d)	Reference	Reference
2nd tertile (71 – 83 g/d)	0.68 [-0.23, 1.59]	0.85 [0.31, 2.28]
3rd tertile (84 – 127 g/d)	-0.49 [-1.39, 0.39]	0.63 [0.22, 1.78]
Model 5: carbohydrate (%)		
1st tertile (31.8 – 53.4)	Reference	Reference
2nd tertile (53.6 – 58.9)	1.32 [0.43, 2.22]**	4.79 [1.66, 13.81]**
3rd tertile (59.1 – 75.3)	0.69 [-0.22, 1.60]	2.11 [0.75, 5.86]
Model 6: protein (%)		
1st tertile (6.6 – 11.4)	Reference	Reference
2nd tertile (11.2 – 14.2)	-0.18 [-1.10, 0.74]	0.49 [0.18, 1.33]
3rd tertile (14.3 – 23.5)	-0.51 [-1.43, 0.42]	0.77 [0.28, 2.09]
Model 7: fat (%)		
1st tertile (14.9 – 27.9)	Reference	Reference
2nd tertile (28 – 33.5)	1.02 [0.13, 1.91]**	2.63 [0.89, 7.80]*
3rd tertile (33.6 – 48.9)	-0.56 [-1.41, 0.30]	0.47 [0.17, 1.27]

[1]n= 111; complete-case analysis
[2]analyses adjusted for father's job exposure risk, no. of young sibling in the household (one or more), father's smoking, CRP-adjusted ferritin levels and child's BMI
[3]liner regression analyses
[4]logistic regression analyses
[5]ntake adjusted for total calorie by residual method
*p< 0.1 **p< 0.05

Table 18: Association between micronutrient intake and blood lead levels in Uruguyan children[1,2,3]

Nutrients	BLL (µg/dL)[4] β [95% CI]	BLL≥ 4.9 µg/dL[5] OR [95% CI]
Model 8- calcium		
1st tertile (125 – 626 mg/d)	Reference	Reference
2nd tertile (633 – 849 mg/d)	-0.34 [-1.32, 0.67]	1.25 [0.39, 4.13]
3rd tertile (850 – 1496 mg/d)	-0.81 [-1.77, 0.15]*	0.24 [0.08, 0.79]**
Model 9- iron		
1st tertile (3.66 – 8.92 mg/d)	Reference	Reference
2nd tertile (8.93 – 10.91 mg/d)	0.21 [-0.56, 2.56]	[0.02, 6.42]
3rd tertile (10.92 – 58.21 mg/d)	-0.19 [-1.13, 0.74]	0.12 [0.01, 1.39]
Model 10 zinc		
1st tertile (0.60 – 3.97 mg/d)	Reference	Reference
2nd tertile (3.98 – 5.92 mg/d)	-0.58 [-1.49, 0.34]	0.57 [0.21, 1.57]
3rd tertile (5.93 – 15.03 mg/d)	-0.23 [-1.18, 0.73]	1.11 [0.40, 3.08]
Model 11- vitamin C		
1st tertile (< 0 – 32.78 µg/d)	Reference	Reference
2nd tertile (33.55 – 58.87 µg/d)	0.69 [-0.24, 1.64]	1.04 [0.35, 3.08]
3rd tertile (59.23 – 306.42 µg/d)	0.30 [-0.63, 1.23]	2.08 [0.71, 6.17]
Model 12- folate		
1st tertile (71.56 – 411.75 µg/d)	Reference	Reference
2nd tertile (414.38 – 527.36 µg/d)	-0.64 [-1.61, 0.34]	0.73 [0.27, 2.02]
3rd tertile (534.53 – 925.86 µg/d)	0.32 [-0.65, 1.28]	1.32 [0.47, 3.67]
Model 13- calcium x iron interaction	---[6]	---[6]
Calcium Iron Calcium x iron		
Model 14- iron x zinc interaction	---[6]	---[6]
Iron Zinc Iron x zinc		
Model 15- iron x vitamin C interaction	---[6]	---[6]
Iron Vitamin C Iron x vitamin C		
Model 16- main effects: calcium & iron		
Calcium		
2nd tertile	-0.57 [-1.60, 0.47]	1.21[0.33, 4.55]
3rd tertile	-0.99 [-1.99, -0.06]**	0.17 [0.04, 0.63]**
Iron		
2nd tertile	-0.04 [-0.98, 0.90]	1.88 [0.53, 6.72]
3rd tertile	0.04 [-0.96, 1.05]	1.23 [0.29, 5.24]
Model 17- main effects: iron & zinc		
Iron		
2nd tertile	0.09 [-0.89, 1.01]	1.16 [0.41, 3.27]
3rd tertile	0.04 [-0.92, 1.01]	0.97 [0.34, 2.78]
Zinc		
2nd tertile	-0.63 [-1.80, 0.10]	0.44 [0.13, 1.47]
3rd tertile	-0.33 [-1.25, 0.65]	1.77 [0.47, 3.34]
Model 18-main effects: iron & vitamin C		
Iron		
2nd tertile	-0.06 [-1.03, 0.88]	1.14 [0.40, 3.26]
3rd tertile	-0.25 [-1.18, 0.79]	0.62 [0.20, 1.89]
Vitamin C		
2nd tertile	0.66 [-0.27, 1.63]	1.02 [0.29, 3.58]
3rd tertile	0.48 [-0.63, 1.30]	2.63 [0.69, 3.92]

[1]n= 111

[2]analyses adjusted for father's job exposure risk, no. of young sibling in the household (one or more), father's smoking, CRP-adjusted ferritin levels and child's BMI.

[3]dietary intake of micronutrients adjusted for total energy intake by residual method. [4]linear regression analyses. [5]logistic regression analyses.

[6]interactions between iron-zinc, iron-vitamin C and iron-calcium were not significant (p> 0.15). Analysis was repeated to test the main effects of micronutrients. *p< 0.1 **p< 0.05

Table 19: Association between macronutrient intake and BLL with imputed dataset[1,2]

Nutrients	BLL (μg/dL) β [95% CI]	BLL≥ 4.9 μg/dL β [95% CI]
Model 1: energy		
1st tertile (937 – 1961 kcal/d)	Reference	Reference
2nd tertile (1973 – 2447 kcal/d)	0.26 [-0.45, 1.17]	0.71 [0.20, 2.12]
3rd tertile (2465 – 4236 kcal/d)	0.23 [-0.73, 1.19]	1.05 [0.37, 2.96]
Model 2: carbohydrate[3]		
1st tertile (194 – 299 g/d)	Reference	Reference
2nd tertile (300 – 332 g/d)	1.12 [0.31, 3.12]**	3.04 [1.72, 8.62]**
3rd tertile (333 – 437 g/d)	0.53 [-0.38, 1.44]	2.20 [0.79, 6.09]
Model 3: protein[3]		
1st tertile (34 – 64 g/d)	Reference	Reference
2nd tertile (65 – 77 g/d)	-0.49 [-1.40, 0.41]	0.85 [0.32, 2.28]
3rd tertile (78 – 338 g/d)	-0.43 [-1.39, 0.54]	0.63 [0.22, 1.79]
Model 4: fat[3]		
1st tertile (19 – 70 g/d)	Reference	Reference
2nd tertile (71 – 83 g/d)	0.68 [-0.23, 1.59]	0.85 [0.31, 2.28]
3rd tertile (84 – 127 g/d)	-0.49 [-1.39, 0.39]	0.63 [0.22, 1.78]
Model 5: carbohydrate (%)		
1st tertile (31.8 – 53.4)	Reference	Reference
2nd tertile (53.6 – 58.9)	1.32 [0.43, 2.22]**	4.79 [1.66, 13.81]**
3rd tertile (59.1 – 75.3)	0.69 [-0.22, 1.60]	2.11 [0.75, 5.86]
Model 6: protein (%)		
1st tertile (6.6 – 11.4)	Reference	Reference
2nd tertile (11.2 – 14.2)	-0.18 [-1.10, 0.74]	0.49 [0.18, 1.33]
3rd tertile (14.3 – 23.5)	-0.51 [-1.43, 0.42]	0.77 [0.28, 2.09]
Model 7: fat (%)		
1st tertile (14.9 – 27.9)	Reference	Reference
2nd tertile (28 – 33.5)	0.09 [0.06, 1.81]**	2.63 [0.89, 7.80]*
3rd tertile (33.6 – 48.9)	-0.51 [-1.41, 0.30]	0.47 [0.17, 1.27]

[1]data presented as β or Odds ratio (OR) [95% confidence interval (CI)]; number of imputed dataset= 10
[2]analyses adjusted for father's job exposure risk, no. of young sibling in the household (one or more), father's smoking, CRP-adjusted ferritin levels and child's BMI
[3]intake adjusted for total calorie by residual method
*p< 0.1 **p< 0.05

Table 20: Association between micronutrient intake and blood lead levels with imputed dataset[1,2]

Nutrients	BLL (µg/dL) β [95% CI]	BLL≥ 4.9 µg/dL β [95% CI]
Model 8- calcium[2]		
1st tertile (125 – 626 mg/d)	Reference	Reference
2nd tertile (633 – 849 mg/d)	-0.34 [-1.67, 0.39]	1.15 [0.39, 4.13]
3rd tertile (850 – 1496 mg/d)	-0.39 [-1.11, 0.15]*	0.30 [0.12, 0.60]**
Model 9- iron[2]		
1st tertile (3.66 – 8.92 mg/d)	Reference	Reference
2nd tertile (8.93 – 10.91 mg/d)	0.21 [-0.56, 2.56]	[0.02, 6.42]
3rd tertile (10.92 – 58.21 mg/d)	-0.19 [-1.13, 0.74]	0.12 [0.01, 1.39]
Model 10- zinc[2]		
1st tertile (0.60 – 3.97 mg/d)	Reference	Reference
2nd tertile (3.98 – 5.92 mg/d)	-0.58 [-1.49, 0.34]	0.57 [0.21, 1.57]
3rd tertile (5.93 – 15.03 mg/d)	-0.23 [-1.18, 0.73]	1.11 [0.40, 3.08]
Model 11- vitamin C[2]		
1st tertile (< 0 – 32.78 µg/d)	Reference	Reference
2nd tertile (33.55 – 58.87 µg/d)	0.69 [-0.24, 1.64]	1.04 [0.35, 3.08]
3rd tertile (59.23 – 306.42 µg/d)	0.30 [-0.63, 1.23]	2.08 [0.71, 6.17]
Model 12- folate[2]		
1st tertile (71.56 – 411.75 µg/d)	Reference	Reference
2nd tertile (414.38 – 527.36 µg/d)	-0.64 [-1.61, 0.34]	0.73 [0.27, 2.02]
Model 13- calcium x iron interaction	---[4]	---[4]
Calcium		
Iron		
Calcium x iron		
Model 14- iron x zinc interaction	---[4]	---[4]
Iron		
Zinc		
Iron x zinc		
Model 15- iron x vitamin C interaction	---[4]	---[4]
Iron		
Vitamin C		
Iron x vitamin C		
Model 16- main effects: calcium & iron		
Calcium		
2nd tertile	-0.57 [-1.60, 0.47]	1.21[0.33, 4.55]
3rd tertile	-0.99 [-1.99, -0.06]**	0.17 [0.04, 0.63]**
Iron		
2nd tertile	-0.04 [-0.98, 0.90]	1.88 [0.53, 6.72]
3rd tertile	0.04 [-0.96, 1.05]	1.23 [0.29, 5.24]
Model 17- main effects: iron & zinc		
Iron		
2nd tertile	0.09 [-0.71, 0.88]	1.16 [0.41, 3.27]
3rd tertile	0.03 [[-0.92, 1.01]	0.97 [0.34, 2.78]
Zinc		
2nd tertile	-0.63 [-1.80, 0.10]	0.44 [0.13, 1.47]
3rd tertile	-0.33 [-1.25, 0.65]	1.77 [0.47, 3.34]
Model 18-main effects: iron & vitamin C		
Iron		
2nd tertile	-0.06 [-0.09, 0.88]	1.14 [0.40, 3.26]
3rd tertile	-0.25 [-1.01, 0.72]	0.62 [0.20, 1.89]
Vitamin C		
2nd tertile	0.66 [-0.27, 1.63]	1.02 [0.29, 3.58]
3rd tertile	0.48 [-0.63, 1.30]	2.63 [0.69, 3.92]

[1] number of imputed dataset= 10

[2]Analyses adjusted for father's job exposure risk, location of the house, no. of young sibling in the household (one or more), father's smoking, CRP-adjusted ferritin levels and child's BMI.

[3]Interactions between iron-zinc, iron-vitamin C and iron-calcium were not significant (p> 0.15). Analysis was repeated to test the main effects of micronutrients.

*p< 0.1 **p< 0.05

Chapter 5

Association of blood lead levels with urinary F_2-8α Isoprostane and 8-hydroxy-2-deoxy-Guanosine concentrations in first-grade Uruguayan children

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ABSTRACT:

Oxidative stress is a potential molecular mechanism for lead-induced toxicities. However, we still have limited understanding of the relation between low-level lead exposure and oxidative stress, especially in children. This study examines the association between blood lead level (BLL) and two oxidative stress markers-urinary F_2-8α Isoprostane (isoprostane; marker of lipid peroxidation) and 8-hydroxy-2-deoxy-Guanosine (8-OH-dG; marker of DNA damage) in ~200 5-8 year old children from Montevideo, Uruguay. The role of dietary intakes of vitamin C and zinc in modifying the relation between BLL and oxidative stress was also examined in this cross-sectional study. The mean (SD) BLL of the study children was 4.7 (2.2) µg/dL, with 30.2% children having BLL ≥5 µg/dL, the current reference level set by the US Centre for Disease Control for identifying, monitoring and management of children with elevated BLL. In covariate-adjusted analysis, there was a weak positive association between BLL and urinary isoprostane (adjusted for specific gravity) [$\beta = 0.08$, $p < 0.1$]. No association was found between children's BLL and urinary 8-OH-dG. Interactions between dietary intakes of vitamin C or zinc and BLL on oxidative stress biomarkers were not consistent. In the main effect model, when BLL and vitamin C were modeled together, BLL significantly predicted isoprostane concentration but vitamin C intake did not. These data suggest a potential adverse association between BLL and oxidative stress in children with low-level lead exposure and indicate a need to further study the effects of lead on other oxidative stress measures, as well as the role of oxidative stress in mediating lead toxicity.

Keywords: lead, isoprostane, 8-hydroxy-2-deoxy-Guanosine, oxidative stress, child, school-age, lipid peroxidation, DNA damage, Uruguay.

1. INTRODUCTION:

Lead exposure poses a threat to children's growth and development during and beyond childhood (Bellinger et al., 2011, Mazumder et al., 2011). Although there may be several mechanisms by which lead exerts its toxic effects, oxidative stress (OS) has been suggested as one molecular mechanism for lead-induced toxicities at the cellular level (Ahamed & Siddiqui, 2007). Generation of an excessive amount of free radicals/pro-oxidants such as reactive oxygen or nitrogen species (ROS or NOS), leading to an imbalance between antioxidant defense and pro-oxidants, can cause damage to vulnerable cellular targets such as unsaturated fatty acid chains in membranes, thiol groups in proteins, and nucleic acid bases in DNA (Stohs & Bagchi, 1995). Lead has the ability to induce oxidative imbalance by multiple mechanisms, including generating ROS, affecting the antioxidant defense system, and altering the structure and functions of cellular membranes (Ahamed & Siddiqui, 2007).

Evidence from experiments in animals suggest that exposure to high levels of lead can induce the generation of lipid peroxidation products such as malonaldehyde (MDA), alter the activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT); and alter the structure of DNA or damage nucleic acid bases in DNA (Berrahal et al., 2011; Haleagrahara et al., 2011; Sandhir et al., 1994). Higher levels of MDA, and structural damage to DNA (higher comet tail length, increased frequency of micronucleus and increased chromosomal damage) have also been reported in occupationally-exposed workers with high BLL compared to adults with lower BLL (Grover et al., 2010; Permpongpaiboon et al., 2011). In addition, lead levels in blood and urine were positively associated with MDA and inversely associated with SOD, CAT, vitamin C, E, carotenoids, and selenium (Se) in occupationally-exposed adults (Garcon et al., 2004; Gurer-

orhan et al., 2004; Oktem et al., 2004; Prokopowicz et al., 2011). Furthermore, among very few studies in non-occupationally exposed adults with low BLL, Lee and colleagues (2006) found a positive association between BLL and serum γ-glutamyltranferase (GGT), a liver enzyme and an early marker of oxidative stress, after adjusting for age, sex, race and socio-economic status.

In contrast, the relationship between BLL and oxidative stress in children is not well studied. Few studies have reported higher levels of oxidative stress in children with high levels of exposure to lead than those with lower exposure (Ahamed et al., 2008; Cabral et al., 2012, Jin et al., 2006). For example, Jin and colleagues (2006) observed significantly higher MDA concentrations in 3 to 6 year old Chinese children with BLL≥ 10 µg/dL than those with BLL< 10 µg/dL. Concentrations of plasma MDA, thio-barburic acid reactive substances (TBARS), activities of antioxidant enzymes (SOD and CAT), and DNA damage were also positively associated with children's BLL in some studies (Ahamed et al., 2011, Diouf et al., 2006; Jaso-Pinada et al., 2012). Except for one (Mielzynska et al., 2006), these analyses were not controlled for factors such as exposure to other metals, age, body-weight, socio-economic or nutritional status, all of which may potentially affects oxidative status. Mielzynska and colleagues (2006) found a significant positive association between BLL and DNA damage, measured by micronuclei (MN) and sister chromatid exchange (SCE) levels in peripheral lymphocytes, after adjusting for age, sex, exposure to environmental tobacco smoke, indoor emission from coal-burning stoves, concentrations of plasma 1-hydroxypyrene (biomarker for Polycyclic hydrocarbon exposure) and parental education in 5-14 year old Polish children with a mean (SD) BLL of 7.7 (4.3) µg/dL. In contrast, no significant association was observed between BLL and activities of the antioxidant enzymes-CAT and SOD in 0 to 14 year old Argentinian children with an average BLL of 2.6 µg/dL (Martinez et al., 2013). The conflicting findings in these two

studies may have to do with a lack of adjustment for potentially influential factors by Martinez and colleagues (2013) or the large difference in mean BLL between the two study groups.

Given the paucity of research among children with respect to low-level lead exposure and oxidative stress, our aim was to examine the association between BLL and two measures of oxidative stress: urinary F_2- 8α isoprostane (isoprostane) and 8-hydroxy- 2 deoxy Guanosine (8-OH-dG) in first-grade children living in Montevideo, Uruguay. Isoprostanes are generated from the oxidation of tissue phospholipids (mostly arachidonic acid) by ROS and have been linked to chronic disease (Basu, 2008; Kanaya et al., 2011; Kaviarason et al., 2009). On the other hand, 8-OH-dG is produced by the hydroxylation of the guanosine moiety of a nucleic acid base by ROS or NOS, and is an established marker of oxidative DNA damage (Valavanidis et al., 2009).

The production of both isoprostane (Chen et al., 2009, Dietrich et al., 2001; Holt et al., 2009; Tomey et al., 2007;) and 8-OH-dG (Hong et al., 2013; Sram et al., 2012; Engstrom et al., 2010) may be affected by nutrients that modulate the biological pathways for the production of free radicals or influence the balance between free radicals and antioxidant capacity in the body. For example, higher intake of *trans* fatty acids and lower intake of zinc were associated with higher isoprostane concentrations in adult US women (Tomey et al., 2007). While *trans* fatty acids stimulate the increased production of free radicals, zinc may exert its protective antioxidant effect by reducing free radical production or acting as a co-enzyme to SOD- a critical enzyme in the antioxidant defense system (Tomey et al., 2007). The intake of vitamin C and folate was also inversely related to isoprostane concentrations in 13-17 year old US children (Holt et al., 2009). Similarly, a limited number of studies reported an inverse association between dietary intake or plasma levels of antioxidants such as vitamin C and E, and 8-OH-dG (Hong et al., 2013; Sram et

al., 2012). Therefore, dietary intakes of both macro and micronutrients may potentially modulate lead-induced oxidative stress.

Dietary information on the intake of total energy, carbohydrate, protein, total fat, fiber, iron, zinc, calcium, folate and vitamin C was available for the study children. We investigated the role of two antioxidants- the intake of vitamin C and zinc on the association between BLL and urinary concentrations of isoprostane, and 8-OH-dG in first-grade children from Montevideo, Uruguay. We tested the hypothesis that higher intake of vitamin C or zinc would attenuate the effects of BLL on oxidative stress measures.

2. METHODS:

2.1. Participants:

All first grade children attending private elementary schools located in different neighborhoods of Montevideo were eligible for the study. The sole exclusion criterion was a previous diagnosis of lead poisoning (BLL >45 µg/dL). None of the children were excluded on this basis. The detailed description of the study setting and predictors of BLL in these children has been provided elsewhere (see Chapter 3). Of the 410 eligible children, 211 finally enrolled into the study. The study was approved by the Ethics Committee for Research Involving Human Participants at the Pennsylvania State University, the Catholic University of Uruguay, and the University of the Republic of Uruguay.

2.2. Parental questionnaire:

Parents/caregivers who agreed to participate in the study were invited for a meeting at the school to fill out a questionnaire about socio-demographic characteristics of the family, the child's medical history and the home environment. The details of the parental questionnaire were provided in chapter 3.

2.3. Anthropometric measurements:

Children were weighed in triplicate using a digital scale (Seca 872, Shorr Productions, Colombia, MD) and their height was measured in triplicate using a portable stadiometer (Seca 214, Shorr Productions, Colombia, MD). A trained pediatric nurse or nutritionist carried out both measurements. A final weight was calculated by averaging the three measurements and subtracting standard weights of children's clothing. A detailed description of anthropometric measurement is provided elsewhere (chapter 4). Based on the mean weight (corrected for clothing) and height measurements, the child's BMI was calculated. Z-scores on weight-for-age, height-for-age and BMI-for-age were calculated using the WHO Anthro software (WHO, 2010). Overweight (BMI-z-score> +1 SD) and obesity (BMI-z-score> +2 SD) were defined according to WHO standards (WHO reference 2007).

2.4. Blood and urine collection:

Fasting blood was collected by a phlebotomy nurse at the school during a morning visit. Blood collection was described in detail previously (chapter 4). Children brought in their first void urine samples on the morning of the clinic visits in cups that had been provided to them on a prior occasion. Because urine samples were intended to be stored for future analysis of metals (Pb, As, Cd), to decrease potential contamination of the sample from the receptacle, each cup was rinsed repeatedly with 10% HNO_3 and deionized water, and then dried before being given to the participants. The urine samples were transported on ice to the CEQUIMTOX at the Faculty of Chemistry at the University of the Republic on the day of the clinic visit. There, urine aliquots for measuring isoprostane were made immediately and stored at -80°C in the presence of 0.005% BHT (Beta hydroxy toluene) to prevent oxidative formation of isoprostane. The BHT solution was prepared by dissolving 5 mg of 3, 5 Di- tert- 4- butylhydroxytoluene (Sigma- Aldrich, Saint

Louis, MO) in 1 ml of ethanol. The rest of the aliquots were stored at -20°C in 10 mL plastic tubes previously rinsed with 10% HNO_3 and deionized water. All urine samples were shipped on dry ice and stored at -80°C (for isoprostane) and -20°C (for 8-OH-dG) until analysis at the Department of Nutritional Sciences, the Pennsylvania State University (USA).

Specific gravity of each urine sample was measured using a portable specific gravity refractometer (PAL 10S, Atago Inc, USA) on the day of the collection. Each day, the refractometer was prepared by pipetting 2-3 drops of tap water onto the prism surface and taking a reading. If the display did not read 1.000, the zero key was pressed. After the zero setting was successfully completed, samples could be tested. Approximately 2-3 drops of a urine sample was placed onto the prism surface and a reading was taken. The measurement value was displayed on the screen and noted down. The sample was wiped off with tissue paper, the prism was then washed with clean water and dried off.

2.5. Blood lead analysis:

Blood lead concentrations were measured at the CEQUIMTOX, at the Faculty of Chemistry of the University of the Republic by Atomic Absorption Spectrometry (AAS, VARIAN SpectrAA-55B) using flame or graphite furnace ionization techniques, depending on the volume of whole blood available. The detection limit was 1.8 µg/dL for flame and 0.8 µg/dL for graphite furnace AAS techniques, respectively. Details on analysis of whole blood concentration were described in chapter 4.

2.6. Hemoglobin measurement:

Description on measurement of hemoglobin was available in chapter 4.

2.7. Serum ferritin and C-reactive protein (CRP) *analysis:*

Serum ferritin concentrations were determined in duplicate by an immunoradiometric assay kit (Coat-A-Count Ferritin IRMA; SIEMENS Diagnostic Products, USA). Concentration of CRP was measured to identify the presence of subclinical inflammation/infection in the study children. CRP was analyzed in duplicate using an ELISA technique described by Erherdt and colleagues (2004). Serum ferritin concentrations were adjusted for the presence of inflammation by adjusting for serum CRP concentration using a method described by Thurnham and colleagues (2010). The analysis of serum ferritin and CRP concentration and adjustment of ferritin for CRP were described in detail previously (chapter 4).

2.8. Urinary isoprostane analysis:

Urinary isoprostane was analyzed at the Pennsylvania State University using a commercially available competitive enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). Prior to this analysis, all samples were purified using a solid phase extraction (SPE) method, to remove any organic solvents that could interfere with the proper measurements of isoprostane. Sample purification before the assay is suggested by the manufacturer to recover the free isoprostane from mixtures of compounds.

Purification of samples was done according to the manufacturer's instruction. Urine samples were thawed at room temperature and vortexed vigorously. Then, one ml of the sample was diluted with 2.5 ml of 1M acetate buffer. For each sample, a 6 ml column (C-18 SPE cartridge, Cayman Chemical, Ann Arbor, MI) was activated by rinsing with 5 ml HPLC-grade methanol and 5 ml of double distilled water. The columns were not allowed to dry. Next, the diluted samples were passed through the activated columns, followed by a rinse with 5 ml of double distilled water and 5 ml HPLC grade hexane. Isoprostane in each sample was eluted with

5 ml ethyl acetate containing 1% methanol. The eluted isoprostane in ethyl acetate solution was stored in a glass vial at -80°C until analysis. On the day of the analysis, samples were brought to room temperature and the ethyl acetate fraction was evaporated under a stream of dry nitrogen. The dried samples were then reconstituted with a buffer provided in the EIA kit. The assay was performed with the reconstituted samples following the manufacturer's instructions. Standards and samples were run in duplicate with an average CV of 5.4%. Each 96-well plate, eight standards and samples were pippetted into individual wells pre-coated with mouse Anti-rabbit IgG. Antiserum (8-isoprostane-specific rabbit antiserum) and 8-iso-$PGF_{2\alpha}$ acetylcholinesterase (AChE) conjugate tracer were added to the wells. This assay in based on the competition between isoprostane and an 8-isoprostane-acetylcholinesterase conjugate for a limited number of isoprostane-specific rabbit antiserum binding sites. Subsequently, the plates were incubated for 18 hours at 4°C. After incubation, the plates were emptied and rinsed with wash buffer provided in the kit. A color reagent which contains the substrate to AChE (Ellman's reagent, Cayman Chemical) was added and the absorbance was read at 412 nm by an automated plate reader (Epoch Microplate Spectrophotometer, Biotek, Winooski, VT). In addition to the standards and samples, the absorbance readings of non-specific binding (NSB), maximum binding (B_0) and total activity (TA) of the tracer were measured. A ratio (B/B_0) was calculated for the absorbance of each standard or sample to that of the B_0 well. Samples were diluted 10 fold to guarantee measurements within the optimum 20-80% B/B_0 range. If samples fell outside 20-80% B/B_0 range, they were rerun with further dilution (if B/B_0 <20%) or without any dilution (if B/B_0 >80%). The concentration of isoprostane was determined by fitting the data to a logarithmic regression line. The regression line was derived from the standard curve plot (concentration of the standards plotted against the logarithmic values of B/B_0). Finally, isoprostane levels were

adjusted to the mean specific gravity (1.023) to correct for variation in fluid intake. The formula used to calculate specific gravity-adjusted isoprostane was:

$$\text{adjusted isoprostane} = \frac{(\text{isoprostane} * 1.023 - 1)}{(\text{specific gravity} - 1)}$$

2.9. Urinary 8-OH-dG analysis:

For the analysis of 8-OH-dG, aliquots of 1 ml of urine were brought to room temperature and thawed urine specimens were centrifuged at 4000 × g for 10 min to remove any sediment. Urinary 8-OH-dG was analyzed by a commercially available competitive enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). Samples were diluted 100 fold with an EIA buffer provided by the manufacturer. Standards and samples were run in duplicate with a CV of 7%. Each 96-well plate, eight standards and samples were pippetted into individual wells pre-coated with mouse Anti-rabit IgG. Both 8-OH-dG monoclonal antibody and 8-OH-dG acetylcholinesterase (AChE) conjugate tracer were added to the wells. This assay is based on the competition between 8-OH-dG and 8-OH-dG tracer for a limited amount of 8-OH-dG monoclonal antibody in each well. The plates were incubated for 18 hours at 4°C. After incubation, plates were emptied and rinsed with wash buffer provided in the kit. A color reagent which contains the substrate to AChE (Ellman's reagent, Cayman Chemical) was added and the absorbance was read at 412 nm by an automated plate reader (Epoch Microplate Spectrophotometer, Biotek, Winooski, VT). Similar to the isoprostane analysis, the absorbance readings of non-specific binding (NSB), maximum binding (B_0) and total activity (TA) of the tracer were measured and the ratio (B/B_0) was calculated for the absorbance of each standard or sample to that of the B_0 well. Samples were diluted 100 fold initially to guarantee measurements within the optimum 20-80% B/B_0 range. If samples fell outside 20-80% B/B_0 range, they were rerun with further dilution (if B/B_0 <20%) or less or without dilution (if B/B_0 >80%). The

concentration of 8-OH-dG was determined by fitting the data to a logarithmic regression line. The regression line was derived from the standard curve plot (concentration of the standards plotted against the logarithmic values of B/B_0).

As indicated by the manufacturer, interference by other urinary components in measuring 8-OH-dG by the assay is infrequent for urine samples. Sample purification has only been recommended in case of a 20% or greater disparity between the concentrations of 8-OH-dG in two different dilutions of the same sample. To test for interference, ten random test samples were diluted to two different levels (varied between samples to fit the data within 20-80% B/B_0 range) and the final concentrations of 8-OH-dG were compared. On average, the concentrations differed by 10% in two different dilutions of the same sample and therefore no purification was done. Specific gravity adjustment was also done for urinary 8-OH-dG levels.

2.10. Dietary assessment:

To determine children's dietary intakes, two 24- hour dietary recalls were conducted by five trained nutritionists with the mother or another caregiver familiar with the child's diet. The child was present at the time and contributed to the recall, particularly with the recollection of food consumed at school. One recall took place at the school on the day of the blood draw and the second recall took place over the phone without prior appointment, at least 2 weeks later. It fell either on a weekday or a weekend. The dietary data collection and the analysis of data collected by two 24-hour diet recalls was described previously (chapter 4).

2.11. Statistical Analysis:

All statistical analyses were performed using STATA 12.0 (StataCorp, College Station, TX, USA).

2.11.1. Selection and descriptions of covariates:

Covariates were chosen based on the previous literature or if they were associated with isoprostane, 8-OH-dG or BLL in bivariate regression analyses (p-value< 0.15). Covariates for the multivariate models with isoprostane as outcome included family possessions of household items (entered as categorical variable), parental involvement for six months or more in jobs with potential lead exposure (categorical variable), crowding at home (continuous variable), mother's smoking status (categorical variable), child's body mass index (BMI: weight (kg)/height (m^2)), CRP-adjusted ferritin and serum concentrations of CRP. For models with 8-OH-dG as an outcome, the covariates were child's age, BMI, CRP-adjusted ferritin values, serum concentrations of CRP, mother's education, and father's involvement in jobs with potential for lead exposure. All the socio-demographic covariates were selected and created from the self-reported parental questionnaire as described before (chapter 3).

2.11.2. Complete case analysis:

A series of univariate and multivariate regression models was built to test the independent and interactive effects of BLL, and intakes of vitamin C and zinc on urinary measures of oxidative stress (isoprostane and 8-OH-dG). First, to test whether lead exposure was independently associated with oxidative stress, BLL was entered as a continuous independent variable in unadjusted and adjusted regression analyses (Model 1), conducted separately for isoprostane and 8-OH-dG. Subsequently, intakes of vitamin C and zinc (both as tertiles) were entered as independent variables (without BLL) in regression analyses to test the relation between these nutrients and oxidative stress (Model 2). For adjusted analyses, all models included the set of covariates described above.

The potential interactions between BLL and intakes of vitamin C, and zinc were tested in separate regression models for isoprostane and 8-OH-dG. To test the interactive effects of BLL and vitamin C, two independent variables (BLL and vitamin C) plus the interaction term (BLL x vitamin C) were included in a model (Model 3). Similarly, BLL, zinc, and the interaction between BLL and zinc were modeled together (Model 4). Interaction terms were created by crossing the individual nutrient tertiles with BLL as a continuous variable (centered at mean value). Non-significant interactions ($p > 0.15$) were removed and regression models were re-run testing the main effects of BLL and vitamin C (Model 5), and BLL and zinc intake (Model 6). The interactions and the main effects were first tested in unadjusted and then in covariate-adjusted multiple regression models. The multivariate models were adjusted for covariates described before.

2.11.3. Missing data analysis:

In the complete-case-analysis, the outcome variables of this study, isoprostane and 8-OH-dG, independent variable including BLL and all the possible predictor variables except for age and sex were subject to missing data. Muiltivariate multiple imputation was used to impute missing variables. The imputation model included the primary outcome variables (isoprostane and 8-OH-dG), blood lead levels, other biomarkers such as hemoglobin and serum ferritin concentrations, and all the socio-demographic variables from all 211 children who completed the study. Interaction terms between individual nutrient tertiles and BLL as a continuous measure were also included in the imputation model. Imputation by chained equations (command "mi ice") of STATA version 12.0 (StataCorp, College Station, TX) was conducted (Royston and White, 2011). Briefly, the algorithm of "mi ice" works in the following manner: first, each variable with missing values is regressed on the other variables in the imputation model. Next,

missing values in each variable are replaced by simulated draws from the posterior predictive distribution of the variable derived from the regression estimation. The process is repeated for all the incomplete variables in the imputation model and the entire round is called a cycle. To stabilize the results, the regression switching cycle is iterated 10 times (default in STATA) and finally a single imputed dataset is generated. In total, 10 imputed datasets were created for the analyses.

3. RESULTS:

3.1. Characteristics of study children:

The socio-demographic, biological and dietary data of the study children are presented in **Table 21**. The mean (standard deviation or SD) age of the children was 6.8 (0.6) years. Urine samples for analyses of isoprostane and 8-OH-dG were available for 186 and 188 children, respectively. In total, 182 children provided blood for lead analysis and information on daily intake of nutrients was available for 187 children. The study children had a mean (SD) BLL of 4.7 (2.2) µg/dL, with 30.2% children having a BLL greater than or equal to 5 µg/dL, the current reference level set by CDC for the identification, monitoring and management of children with elevated BLLs. The mean (SD) urinary isoprostane and 8-OH-dG concentrations, adjusted to specific gravity, were 1.5 (1.2) ng/ml and 48.8 (33.1) ng/ml, respectively. All children had urinary concentrations of isoprostane and 8-OH-dG above the limits of detection. Only 3% of the study participants had anemia (Hb< 11.5 g/dL), whereas more than half of the children had iron-deficiency (CRP-adjusted serum ferritin <15 ng/ml). The mean (SD) body mass index (BMI) was 16.9 kg/m^2, with 19.8 % of the children each being overweight (BMI-z-score> +1 SD) and obese (BMI-z-score> +2 SD) according to WHO standards (WHO reference 2007).

More than half of the mothers (65.7%) reported having some secondary education. Majority of the parents indicated that they were not engaged in jobs with potential for exposure to lead, such as jobs that involve chemicals or metals, recycling, auto-repair, plumbing and paint work for six months or more. However, more than half of the fathers reported employment in jobs that could be a source of exposure to metals (construction, factory work, print shop, mechanic and driver). Mean number of luxury items in the households of the study children were 3.6 out of six items (video player, computer, car, refrigerator, washing machine and home phone), with 42.6% households having less than or equal to three items. About 36.4% of the families did not own a house and 22.6% of the families had more than two people living in one room.

Mean (SD) calorie intake, averaged over two-24 hour recalls, was 2279 (602) Kcal. Mean (SD) energy-adjusted vitamin C and zinc intakes were 58 (53) mg/day and 5 (2) mg/day, respectively. About 34.2% children did not meet the Estimated Dietary Reference Intake (EAR) set by Institute of medicine (IOM, USA) for zinc intake of 4.1 mg/day. Finally, 19.3% of study children did not meet the EAR of 25 mg/day for vitamin C.

3.2. Unadjusted association between blood lead levels, oxidative stress measures and nutrient intake (vitamin C and zinc):

When the independent association between BLL and oxidative stress was assessed in an unadjusted regression analysis, there was a weak positive association between BLL, and isoprostane (**Table 22,** Model 1). Specifically, each 1 µg/dL of BLL was associated with 0.08 ng/ml increase in isoprostane ($p < 0.1$). No association was observed between BLL and 8-OH-dG.

When the independent association between nutrient intake (vitamin C and zinc) and the two oxidative stress measures was tested in unadjusted models, the intakes of vitamin C and zinc

were not associated with isoprostane (Table 22, Model 2). On the other hand, in the model with 8-OH-dG as an outcome, higher zinc but not vitamin C intake was associated with lower levels of 8-OH-dG (Table 22, Model 2). Specifically, compared to children in the lowest tertile of zinc intake, children in the highest tertile had about 20 ng/ml lower 8-OH-dG concentration ($p< 0.01$) (Table 22).

When the BLL-by-nutrient intake interactions were entered into the unadjusted regression models, two interactions were significant (Table 22, Models 3 & 4, $p< 0.15$). The interaction between BLL and vitamin C was significant for 8-OH-dG for the second tertile of vitamin C intake, suggesting that the regression slopes for 8-OH-dG differed between tertile 1 and tertile 2 (Model 3). For these children in the second tertile of vitamin C, there was a 5.7 ng/ml higher 8-OH-dG concentration for every 1 µg/dL increase in BLL ($p< 0.05$) (Model 3). The interaction between BLL and zinc was significant for isoprostane in the second terile of zinc intake, suggesting that an increase in BLL was associated with higher isoprostane levels among children in the second tertile of zinc intake than those who were in the lowest tertile [$\beta= 0.30$; $p< 0.05$] (Model 4).

Because the BLL-by-nutrient intake interactions were not consistent, we finally tested the main effects of BLL and vitamin C tertiles (Model 5) and main effects of BLL and zinc tertiles on oxidative stress measures (Model 6). In the unadjusted main effects models, BLL was significantly associated with isoprostane, but not with 8-OH-dG (Table 22). Vitamin C or zinc intake were not associated with isoprostane in the unadjusted main effect models. When the main effects of BLL and zinc intake were tested, children in the highest tertile of zinc had lower 8-OH-dG concentration than children in the lowest tertile of zinc in [$\beta= -17.12$, $p< 0.01$] (Table 22, Model 6).

3.3. Covariate adjusted association between blood lead levels, oxidative stress measures and nutrient intake (vitamin C and zinc):

In the multivariate regression model, each 1 µg/dL increase in BLL was associated with a 0.08 ng/ml increase in isoprostane concentration ($p< 0.1$) (**Table 23**, Model 1). No significant association was observed between BLL and 8-OH-dG concentrations in multivariate models (Table 23).

When the intakes of vitamin C and zinc were tested in models adjusted for covariates to test the independent association between the two nutrients and BLL, there was a weak inverse association between zinc intake and 8-OH-dG (Table 23, Model 2). Specifically, compared to the lowest tertile of zinc intake, children in the highest tertile had about 13 ng/ml lower 8-OH-dG concentration ($p< 0.1$).

Two interactions, although weak, remained significant ($p< 0.15$) even in the adjusted models: one between BLL and vitamin C intake on 8-OH-dG and another between BLL and zinc on isoprostane for the second tertiles of vitamin C and zinc intake, respectively (Table 23, Models 3 & 4). The predicted increase in 8-OH-dG concentration with 1 µg/dL increase in BLL was higher by 5.20 ng/ml among children who were in the second tertile of vitamin C than those in the lowest tertile ($p= 0.11$) (Table 23, Model 3). Similarly, the predicted increase in isoprostane concentration with 1 µg/dL increase in BLL was higher among children who were in the second tertile of zinc than those in the lowest tertile [$\beta= 0.17$; $p= 0.1$] (Table 23, Model 4).

Since the interactions between BLL and nutrient intake tertiles were inconsistent, the analyses were repeated testing the main effects of BLL and vitamin C, and BLL (Table 23, Model 5) and zinc tertiles (Table 23, Model 6). In the main effects model, each 1 µg/dL increase in BLL was associated with 0.10 ng/ml and 0.09 ng/ml increase in isoprostane concentration

when modelled together with vitamin C (Table 23, Model 5) or zinc (Table 23, Model 6) ($p< 0.05$). There was no association between BLL and 8-OH-dG in the main effect models. Nutrient intakes were not associated with isoprostane or 8-OH-dG in the covariate-adjusted main effects model (Table 23, Models 5 & 6).

3.4. Missing data analysis:

In the covariate-adjusted regression models, with multiple imputed datasets, there was no statistically significant association between BLL and urinary isoprostane or 8-OH-dG concentrations (**Table 24**). Zinc intake was inversely associated with the concentrations of 8-OH-dG, such that compared to the lowest tertile of zinc, children in the highest tertile had about 15 ng/ml lower 8-OH-dG levels ($p< 0.05$) (Table 24). Only one interaction was significant in the imputed dataset: the interaction between BLL and zinc on isoprostane. The interaction effect was similar to the findings of the complete-case analysis, i.e. for an increase in BLL, the increase in isoprostane concentration was higher among children who were in the second tertile than those in the lowest tertile of zinc intake [$\beta= 0.19$; $p< 0.1$] (Table 24).

4. DISCUSSION:

In a study of first-grade children from Montevideo, Uruguay, we examined the relation between children's blood lead levels (BLLs) and oxidative stress measured as urinary concentrations of isoprostane (lipid peroxidation product) and 8-OH-dG (DNA damage marker), and explored the potential interactions of antioxidants such as vitamin C or zinc and BLL on children's oxidative stress. We found a weak positive association between children's BLL and urinary isoprostane after adjusting for socio-demographic and biological factors. In contrast, there was no statistically significant association between BLL and concentration of urinary 8-

OH-dG. We did not find significant meaningful interactions between BLL and intakes of vitamin C and zinc.

Ours is one of the few studies in children to test whether low-level lead exposure is associated with oxidative stress and whether nutritional factors attenuate this association. A recent study by Martinez and colleagues (2013), also tested the association between low-level lead exposure and oxidative stress measured as the activities of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in 0 – 14 year old Argentinian children with a mean (SD) BLL of 2.6 (0.3) µg/dL. They did not find any statistically significant relation between children's BLL and CAT or SOD activities. However, they made no adjustments for socio-demographic, biological or nutritional factors, which may influence their findings. Previously, there were few reports of a positive relation between BLL and oxidative stress, measured as lipid peroxidation in children (Cabral et al 2012, Ahamed et al 2011, 2008, 2006, and 2005). For example, Cabral and colleagues (2012) reported a positive correlation between BLL and plasma malonaldehyde (MDA) concentration (marker of lipid peroxidation) in 1-16 year old children living near a landfill. However, the authors did not perform any adjusted analysis to take into account the other possible predictors of oxidative stress or BLL. Moreover, in the case-control study by Cabral and colleagues, children's BLL ranged from 3.25 µg/dL to 37.34 µg/dL, which is higher than BLL observed in most general pediatric populations including our study (BLL range: 0.8 – 13.2 µg/dL). In contrast, although a cross-sectional study in 3 to 6 year old preschool children with a mean (SD) BLL of 6.98 (1.75) µg/dL found a significantly higher plasma MDA concentrations in children with BLL$\geq$ 10 µg/dL than children with BLL$<$ 10 µg/dL (Jin et al 2006), there was no dose-response relation between BLL and MDA concentrations. The authors attributed the null finding to the induction of low to moderate levels of free radicals at a low BLL

(< 10 µg/dL) which may be compensated by the antioxidant defense system, thus attenuating the extent of oxidative damage. Notably, in their statistical analysis, Jin and colleagues did not report of any adjustment for covariates such as intake/status of antioxidants.

On the other hand, a positive association between lead exposure and lipid peroxidation has been observed in studies with occupationally exposed adults (Garcon et al., 2007; Gurer-Orhan et al., 2004; Khan et al., 2008; Oktam et al., 2004; Permpongpaiboon et al., 2011) and in few reports in the general adult population (Ahamed et al., 2009; Serafim et al., 2012). For example, Permpongpaiboon and colleagues (2011) reported significant correlation between BLL and plasma MDA, total peroxides and conjugated diene in 60 earthenware-factory workers (Mean BLL: 31.4 µg/dL) and their sex-and-age matched controls (Mean BLL: 3.9 µg/dL). In 17-40 year old healthy pregnant women, Sirfim and colleagues (2012) observed that lead levels in the placenta were positively correlated with lipid peroxidation (LPO)- expressed as a sum of MDA and 4-hydroxyalkenals (4-HNE) per gram of total protein. Similarly, placental lead levels were positively related to placental MDA levels in Indian pregnant women (Ahamed et al., 2009). On the other hand, Pollack and colleagues (2012) did not observe any relation between BLL and lipid peroxidation markers such as plasma F_2-8α isoprostane, 9-hydroxy-10,12-octadecadienoic acid (9-HODE), 13-hydroxy-9,11- octadecadienoic acid (13-HODE) and TBARS concentrations in 18-44 years healthy premenopausal women with a median (range) BLL of 0.86 (0.67- 1.20) µg/dL after adjusting for relevant covariates such as age, BMI, smoking status and race. That study did include measures of income, education, physical activity, parity, dietary iron, shellfish, vegetables, dietary selenium, dietary calcium, and total energy intake as potential confounders but these did not alter the effect estimates.

Our finding of a null association between blood lead level and 8-OH-dG, a DNA damage marker, is consistent with the available literature among adults. In occupationally exposed workers from a glass factory, Lin and colleagues (2012) did not find any significant association between urinary lead levels and 8-OH-dG after adjusting for age, smoking status and alcohol consumption. Similarly, BLL was not associated with urinary 8-OH-dG in Bangladeshi pregnant women (Engstrom et al., 2010). To our knowledge, no studies in children examined the association between BLL and 8-OH-dG to compare with our findings. However, three studies in children examined the relation between BLL and DNA damage or DNA repair as measured by the comet assay (also known as single cell gel electrophoresis) or other methods (such as micronuclei (MN) and sister chromatid exchange (SCE) levels in peripheral lymphocytes) (Jasso-Piñeda et al., 2012, Mendez et al., 2008, Mielzynska et al., 2006). Among these three, two studies found higher levels of DNA damage in children with higher BLL compared to children with lower BLL (Jasso-Piñeda et al., 2012, Mendez et al., 2008). Mielzynska and colleagues (2006) observed a direct association between BLL and DNA damage as measured by micronuclei levels in peripheral lymphocytes in 5-14 year old Polish children with a mean BLL of 7.7 µg/dL. While comet assay or other methods used by other studies may be more direct measures of DNA damage, 8-OH-dG is an established marker of oxidative DNA damage and has been used in many studies in children (Dziaman et al 2007; Svecova et al 2009, Szaflarska-Poplawska et al., 2010). In this study, we did not monitor the extent of air pollution or ETS, which have been found to affect 8-OH-dG concentrations (Svecova et al., 2009). However, we tested the relation between self-reported smoking status of the parents, a crude measure of ETS, and children's urinary 8-OH-dG levels. Having parents who smoked was associated with higher levels of 8-OH-dG than having parents who did not smoke, but this association failed to reach

statistical significance. It is possible that low-level lead exposure, such as observed in our study, does not induce significant production of 8-OH-dG or that there may be other unmeasured factors, such as exposure to other toxicants which could better explain the variation in 8-OH-dG concentrations of this group of children.

Although, we did not find significant associations between the intake of vitamin C and isoprostane or 8-OH-dG, there was an inverse relation between zinc intake and 8-OH-dG. In the covariate-adjusted model children in the highest tertile of zinc intake had about 13 ng/ml less 8-OH-dG than children in the lowest tetile of zinc intake. Children who did not meet RDA for zinc intake for the age group (4 mg/day) also had higher 8-OH-dG than children who met the RDA. Zinc has antioxidant properties and provides defense against oxidative stress indirectly (Bray & Betiger, 1989, Powell, 2000). Marginal dietary zinc deficiency has been shown to increase oxidative stress, impair DNA integrity, and increase DNA damage in rats (Nair et al., 2005; Song et al., 2009; Yosef et al., 2002). Notably, majority of the children in our study met the recommended dietary allowance (RDA) for the age group except for zinc (34.2% did not meet the RDA) and to some extent vitamin C (19.3% did not meet RDA).

Our findings need to be considered in light of some limitations. First, many variables, particularly socio-demographic variables, were subject to missing data due to non-response. Therefore the sample size was comparatively small in the final unimputed multivariate models. Our inconsistent findings on interaction between BLL and nutrient may also be due to lack of power. However, we repeated the analyses in multiply imputed datasets to handle the problem of missing data and our results were consistent with complete-case estimates. Nonetheless, our findings need to be tested in a larger sample in future studies. Second, we did not have information on children's exercise routine or physical activity levels, and could not control for

those factors in the analysis. Physical activity may influence oxidative status in the body (Llorente-Cantarero et al., 2012; Tomey et al., 2007) and both inadequate physical activity (Llorente-Cantarero et al., 2012) and acute exercise (Jenkins, 2000) may increase oxidative stress. However, we did adjust for other physical measures such as BMI. Third, we used ELISA methods for analyses of isoprostane and 8-OH-dG concentrations in urine. Chromatographic methods (HPLC or GC-mass spectrometry) are considered a gold standard for measuring 8-OH-dG, and a consistently high correlation has been observed among the urinary 8-OH-dG values analyzed by chromatographic methods and ELISA (Yoshida et al., 2002). Finally, we collected nutrient intake data, but except for iron, we did not have data on biomarkers for those nutrients. As with other diet assessment methods, dietary recalls are subject to memory and respondent bias. To reduce this, we facilitated recall by probing about snacks and specific meals. Despite these limitations, our study has several strengths. This is one of the few studies in children to test the relation between BLL and oxidative stress at a level of lead exposure normally observed in the general population. This is important because the relevance of Pb-induced oxidative stress in general pediatric population with low environmental exposure to lead is not clear. Most of the studies have typically been conducted at higher doses than the concentrations observed in the general population. Next, unlike many previous studies, we made extensive covariate adjustments in our regression models to better explain the variability in children's oxidative stress measures. Third, we evaluated the potential interactions between two antioxidants and BLL on oxidative stress. These interactions have not been tested in previous studies.

5. CONCLUSION:

In 5-8 year old Uruguayan children, we found some evidence of an association between BLL and urinary isoprostane, but not urinary 8-OH-dG. Dietary intakes of vitamin C and zinc did not modify the relation between BLL and oxidative stress. More studies are required particularly in children with low-level environmental lead exposure to confirm our results. In future, research should focus on more detailed investigation of oxidative stress measures including the status of antioxidant enzymes, non-enzymatic antioxidants, along with lipid peroxidation, DNA and protein damage products. This will give a comprehensive understanding of the nature and extent of damage. In addition, studies should examine whether oxidative stress is a potential mediator in the relation between lead exposure and adverse biochemical consequences such as inflammation or functional outcomes such as neurodevelopmental problems, hypertension and diabetes. Furthermore, the role of dietary intake of antioxidant nutrients such as vitamin A, E, C, selenium, and zinc along with specific food groups in protecting against lead-induced oxidative stress should be investigated.

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Table 21: Characteristics of study participants[a]

Variables	N	Mean ± SD or %	Range
Biological factors			
Age (y)	204	6.8 ± 0.6	4.8 – 8.6
Sex (female)	211	43.1%	
Blood lead concentration (µg/dL)	182	4.7 ± 2.2	0.8 -13.2
≥ 5 µg/dL		30.2%	
Hemoglobin (g/dL)	184	13.4 ± 1.1	10.1 – 16.3
<11.5 g/dL		3.0%	
Serum ferritin (ng/ml)	174	14.3 ± 13.2	1.1 – 95.6
< 15 ng/ml		61.2%	
Body Mass Index (kg/m^2)	187	16.89 ± 2.74	7.24 – 26.58
Overweight		19.8%	
Obese		19.8%	
Oxidative stress measures			
Urinary isoprostane (ng/ml)[†]	186	1.5 ± 1.2	0.02 – 7.1
Urinary 8-OH-dG (ng/ml)[†]	188	48.8 ± 33.1	5.0 – 187.6
Socio-demographic characteristics			
Mother's education	183		
Some primary		20.2%	
Some secondary		65.6%	
College or post-graduate		14.2%	
Mother's occupation	143		
Unemployed/stay at home		38.5%	
Domestic help		35.0%	
Factory work		9.8%	
Health work or police		6.3%	
Administration/professional work		10.5%	
Fathers with job exposure risk[b]	131	51.9%	
Parental occupational exposure score[c]	194	0.6 ± 1.1	0 - 8
No involvement		66.9%	
Family possessions of household items[d]	184	3.6 ± 1.4	0 - 6
≤ 3 items		42.6%	
Families do not own home	154	36.4%	
Families with more than 2 person per room	155	22.6%	
Child lives with			
Both parents		69.5%	
Single parent/others[e]		30.5%	
Dietary intake			
Energy (Kcal/day)	182	2279 ± 602	937 - 4236
Zinc (mg/day)	182	5 ± 2	0.6 - 15
< RDA (4 mg/day)[≠]		34.2%	
Vitamin C (mg/day)	182	58 ± 53	0.5 - 342
< RDA (25 mg/day)[≠]		19.3%	

[a]n=211
[b]has exposure risk if employed in jobs such as construction, factory work, print shop, mechanic and driver
[c]involvement in activities that require handling of chemicals or metals, recycling, auto-repair, plumbing and paint work for 6 months or more; score derived by adding all the responses (0= none of the parents, 1= one of the parents, 2= both parents involved); no involvement if score=0 and involvement if score ≥1
[d]items include video player, computer, car, refrigerator, washing machine and home phone
[e]others include grandparents, maternal or paternal uncles or aunts
[f]intakes are adjusted for total calorie intake by residual method
[†]Adjusted for specific gravity (average: 1.023)
[≠]RDA: Recommended dietary allowance set by US-Institute of Medicine

Table 22: Unadjusted association between BLL, intakes of vitamin C, zinc and oxidative stress measures

Models[a]	Oxidative stress measures	
	Isoprostane (ng/ml)	**8-OH-dG (ng/ml)**
	β [95% CI]	β [95% CI]
Model 1- BLL		
BLL	0.08 [-0.001, 0.16]*	-0.15 [-2.42, 2.11]
Model 2- nutrient intake		
Vitamin C		
1st tertile	Reference	Reference
2nd tertile	0.02 [-0.42, 0.46]	8.04 [-5.41, 21.48]
3rd tertile	-0.15 [-0.59, 0.28]	1.17 [-12.05, 14.39]
Zinc		
1st tertile	Reference	Reference
2nd tertile	-0.09 [-0.53, 0.34]	-3.85 [-17.16, 9.46]
3rd tertile	-0.11 [-0.55, 0.33]	-20.84 [-34.27, -7.42]***
Model 3- BLL x vitamin C interaction	---[1]	
BLL		-2.95 [-6.75, 0.83]
Vitamin C:1st tertile	Reference	Reference
2nd tertile		-19.86 [-46.94, 7.22]
3rd tertile		-4.25 [-35.45, 15.60]
BLL x vitc 2nd tertile		5.67 [0.45, 10.89]**
BLL x vitc 3rd tertile		1.57 [-4.69, 7.85]
Model 4- BLL x zinc interaction		---[2]
BLL	0.01 [-0.10, 0.12]	
Zinc:1st tertile	Reference	
2nd tertile	0.04 [-0.39, 0.47]	
3rd tertile	-0.03 [-0.46, 0.39]	
BLL x zinc 2nd tertile	0.30 [0.11, 0.49]**	
BLL x zinc 3rd tertile	0.01 [-0.19, 0.21]	
Model 5- main effects: BLL & vitamin C		
BLL	0.09 [0.01, 0.18]**	0.24 [-2.54, 2.06]
Vitamin C		
1st tertile	Reference	Reference
2nd tertile	-0.06 [-0.50, 0.37]	3.85 [-8.47, 6.18]
3rd tertile	-0.25 [-0.68, 0.19]	0.45 [-11.63, 2.45]
Model 6- main effects: BLL & zinc		
BLL	0.09 [0.01, 0.17]**	-0.16 [-2.43, 2.11]
Zinc		
1st tertile	Reference	Reference
2nd tertile	0.01 [-0.43, 0.44]	-2.80 [-15.22, 9.61]
3rd tertile	-0.02 [-0.46, 0.41]	-17.12 [-29.31, -4.90]***

[a]Model 1: N= 173 (isoprostane), N= 175 (8-OH-dG); Models 2-5: N= 164 (isoprostane), N= 166 (8-OH-dG).

[1]BLL by vitamin C (tertiles) interaction terms was not significant for isoprostane (p> 0.1). Results of regression testing the main effects of BLL and vitamin C are reported under Model 5.

[2]BLL by zinc (tertiles) interaction terms was not significant for 8-OH-dG.

***p< 0.01 **p< 0.05 *p< 0.1

Table 23: Covariate-adjusted association between BLL, oxidative stress measures and intakes of vitamin C and zinc[a]

Models	Oxidative stress measures	
	Isoprostane (ng/ml)[b] β [95% CI]	**8-OH-dG(ng/ml)[c]** β [95% CI]
Model 1- BLL[d]		
BLL (µg/dL)	0.08 [-0.004, 0.17]*	1.16 [-1.64, 3.62]
Model 2- nutrient intake[e]		
Vitamin C		
1st tertile	Reference	Reference
2nd tertile	-0.26 [-0.74, 0.21]	4.60 [-11.06, 20.37]
3rd tertile	-0.23[-0.69, 0.23]	11.24 [-4.25, 26.73]
Zinc		
1st tertile	Reference	Reference
2nd tertile	-0.12 [-0.61, 0.37]	-5.33 [-20.39, 8.75]
3rd tertile	-0.21 [-0.71, 0.29]	-12.99 [-28.76, 1.88]
Model 3- BLL x vitamin C interaction[e]	---[1]	
BLL		-0.94 [-5.66, 3.77]
Vitamin C:1st tertile		Reference
2nd tertile		7.32 [-7.33, 16.48]
3rd tertile		11.08 [-3.45, 25.61]
BLL x vitc 2nd tertile		5.20 [-1.14, 11.55][#]
BLL x vitc 3rd tertile		0.93 [-6.72, 8.57]
Model 4- BLL x zinc interaction[e]		---[2]
BLL	0.05 [-0.07, 0.18]	
Zinc: 1st tertile	Reference	
2nd tertile	0.19 [-0.26, 0.65]	
3rd tertile	0.03 [-0.42, 0.48]	
BLL x zinc 2nd tertile	0.17 [-0.03, 0.38][#]	
BLL x zinc 3rd tertile	-0.01 [-0.22, 0.20]	
Model 5- main effects: BLL and vitamin C[e]		
BLL	0.10 [0.01, 0.18]**	2.14 [-0.84, 5.12]
Vitamin C		
1st tertile	Reference	Reference
2nd tertile	-0.24[-0.70, 0.22]	6.73 [-7.85, 9.33]
3rd tertile	-0.28[-0.72, 0.16]	10.07 [-4.27, 14.42]
Model 6- main effects: BLL and zinc[e]		
BLL	0.09 [0.01, 0.18]**	2.16 [-0.82, 5.14]
Zinc		
1st tertile	Reference	Reference
2nd tertile	0.19 [-0.29, 0.63]	0.77 [-13.24, 14.80]
3rd tertile	0.06 [-0.45, 0.47]	-8.81 [-23.31, 5.69]

[a]complete-case analysis.
[b]models adjusted for family possessions of luxury items, parental involvement of jobs with potential for metal exposure, crowding at home, mother's smoking (yes vs no), body mass index (weight (kg)/ height (m^2)), CRP-adjusted ferritin and CRP concentrations.
[c]models adjusted for child's age, mother's education, father's involvement in jobs with potential for metal exposure, BMI, CRP-adjusted ferritin and CRP values.
[d]Model 1: N= 138 (isoprostane), N= 124 (8-OH-dG
[e]Models 2-6: N= 132 (isoprostane), N= 120 (8-OH-dG)
[1]BLL by vitamin C (tertiles) interaction terms was not significant for isoprostane (p> 0.1). Results of regression testing the main effects of BLL and vitamin C are reported under Model 5.
[2]BLL by zinc (tertiles) interaction terms was not significant for 8-OH-dG.
**p< 0.05 *p< 0.1 [#]p=0.1

Table 24: Covariate-adjusted association between BLL, oxidative stress measures and intakes of vitamin C and zinc using multiply imputed dataset[a]

Models	**Oxidative stress measures**	
	Isoprostane (ng/ml)[b] β [95% CI]	**8-OH-dG(ng/ml)[c]** β [95% CI]
Model 1- BLL		
BLL (µg/dL)	0.04 [-0.04, 0.12]*	0.21 [-3.24, 2.83]
Model 2- nutrient intake		
Vitamin C		
1st tertile	Reference	Reference
2nd tertile	0.03 [-0.41, 0.47]	6.30 [-7.57, 20.17]
3rd tertile	-0.08[-0.51, 0.34]	1.30 [-12.08, 14.70]
Zinc		
1st tertile	Reference	Reference
2nd tertile	-0.08 [-0.50, 0.34]	-1.30 [-14.61, 12.01]
3rd tertile	-0.14 [-0.01, 0.46]	-14.84 [-28.42, -1.26]**
Model 3- BLL x vitamin C interaction	---[1]	---[1]
BLL		
Vitamin C: 1st tertile		
2nd tertile		
3rd tertile		
BLL x vit C 2nd tertile		
BLL x vit C 3rd tertile		
Model 4- BLL x zinc interaction		---[2]
BLL	0.05 [-0.07, 0.18]	
Zinc: 1st tertile	Reference	
2nd tertile	-0.90 [-1.86, 0.06]*	
3rd tertile	-0.23 [-1.22, 0.77]	
BLL x zinc 2nd tertile	0.19 [-0.003, 0.38]*	
BLL x zinc 3rd tertile	0.02 [-0.17, 0.21]	
Model 5- main effects: BLL & vitamin C		
BLL	0.04 [-0.04, 0.12]	-0.35 [-3.30, 2.73]
Vitamin C		
1st tertile	Reference	Reference
2nd tertile	0.04[-0.40, 0.47]	9.36 [-4.57, 23.30]
3rd tertile	-0.08[-0.51, 0.34]	3.29 [-10.21, 16.80]
Model 6- main effects: BLL & zinc	---[3]	
BLL		
Zinc		
1st tertile		Reference
2nd tertile		-1.41 [-14.82, 11.99]
3rd tertile		-15.72 [-29.18, -2.26]**

[a]number of imputated dataset= 10

[b]models adjusted for family possessions of luxury items, parental involvement of jobs with potential for metal exposure, crowding at home, mother's smoking (yes vs no), body mass index (weight (kg)/ height (m^2)), CRP-adjusted ferritin and CRP concentrations.

[c]models adjusted for child's age, mother's education, father's involvement in jobs with potential for metal exposure, BMI, CRP-adjusted ferritin and CRP values.

[1]BLL by vitamin C (tertiles) interaction terms was not significant for isoprostane or 8-OH-dG ($p > 0.1$). Results of regression testing the main effects of BLL and vitamin C are reported under Model 5.

[2]BLL by zinc (tertiles) interaction terms was not significant for 8-OH-dG ($p > 0.1$) Results of regression testing the main effects of BLL and zinc are reported under Model 6.

[3]BLL by zinc interaction was significant for isoprostane. Results of regression testing the interaction effects are reported under Model 4.

**$p < 0.05$ *$p < 0.1$

Chapter 6

DISCUSSION

Due to a vast amount of research on lead, we have extensive knowledge of lead exposure, and especially the health consequences of lead toxicity. Yet, many questions remain unanswered, such as: (1) what are the biological mechanisms through which lead exerts its toxic effects; (2) whether children with nutritional deficiencies are more vulnerable to lead exposure and/or its effects; (3) what are the roles of nutrients and overall what is an effective dietary strategy to reduce exposure and toxicities; (4) what constitutes the most useful cost-effective method to prevent or treat lead exposure particularly at low range of environmental exposure.

The purpose of the current study was to identify the socio-demographic factors and household behaviors predictive of BLL in the study children, to examine the relation between dietary nutrient intake and blood lead level (BLL), and investigate the association between children's BLL and oxidative stress (measured as urinary concentration of F_2- 8α isoprostane or isoprostane and 8-hydroxy- 2-deoxy Guanosine or 8-OH-dG). This cross-sectional study was conducted in 5-8 year old children attending nine primary schools in Montevideo, Uruguay. The most salient socio-demographic and household factors associated with BLL in these children were fathers' employment in jobs with potential for metal exposure (such as construction, factories, print shops, mechanics or drivers), having more than one young child at home and fathers' smoking. Among dietary factors, carbohydrate and energy intake from fat were positively while calcium intake was inversely associated with children's BLL. Finally, there was a weak positive association of children's BLL with urinary isoprostane concentration, but no relation was found between BLL and 8-OH-dG concentration.

With the average BLL of 4.7 µg/dL and 30.2% children having elevated BLL [≥ 5 µg/dL, the current reference level set by Centre for Disease Control; CDC, 2012], the BLL of the study children is comparable to BLLs reported in children of similar age-group from other Latin American countries (Charalambous et al 2009; Beltramino et al 2007; Olivero-Verbal et al 2004) The mean BLL in this study was, however, lower than the BLL observed in a study conducted in 2007 in preschool children living in and around the same neighborhoods of Montevideo (Queirolo et al 2010). Because this study was conducted a few years after the study in preschoolers (more time has passed since the withdrawal of lead from gasoline) and because the study children were older than the preschoolers (young children typically have higher BLL), our finding of lower BLL in the school children is not surprising. Nevertheless, in light of the mounting evidence that lead exposure, even at BLL< 5 µg/dL, is detrimental to children's health, the estimates of BLL of the study children highlight the fact that lead exposure is still a problem in Montevideo.

The socio-demographic and household risk factors (father's smoking, employment, and number of young children in a household) for BLL in the study children were identified through information provided by parents who completed a self-reported questionnaire. The fathers in our study who had the potential for occupational exposure worked in construction, factories, and print shops, and as mechanics or drivers. It is possible that dust contaminated by lead settled on fathers' skin and clothing, which later served as sources of exposure to the children at home (Levin et al 2008). Another predictor of school children's BLLs—the number of young children (<5 years old) at home, as well as fathers' jobs—may reflect the socio-economic status of the family. Families with better socio-economic conditions are less likely to be engaged in activities that will put them at risk for lead exposure. Moreover, families with better resources are more

likely to live in areas with fewer industries (Tong et al, 2000), have better housing conditions (Rauth et al 2008), have higher parental education, and practice behaviors protective of child's health (Pampel et al 2010). On the other hand, 42% of the fathers and 34% of the mothers in this study reported to be current smokers. Since the majority of the study children lived with their parents, it is possible that they were exposed to second-hand smoke at home. It has been shown that tobacco smoke contains lead, which can be inhaled and absorbed though the lungs (Galazyn-Sidorczuk et al 2008; ATSDR 2007; Kalcher et al 1993). Although numerous studies have previously identified socio-demographic factors that can affect children's BLL, dearth of information specifically related to Uruguayan school children makes our findings particularly important and can be used to sensitize parents and health-care providers on the issue of lead exposure, as well as inform policies, initiate preventive measures or target intervention programs. For example, policies could be adopted to build smoke-free environment particularly in schools, playgrounds and other public places. Increasing awareness at workplace to reduce take-home exposure and interventions including educating parents, teachers and children to change behaviors and life-style could be initiated.

This study also examined the relation between dietary intake of nutrients and children's BLLs, and found that the intakes of two macronutrients--carbohydrate (both as absolute amount and as percentage of energy) and fat (as energy %)—were positively associated with children's BLL. However, there was no clear dose-response relationship between these macronutrients and BLL. Specifically, children in the second tertile had higher BLL than those in the first or third tertile of dietary carbohydrate (absolute value or percent energy) and fat percent. We failed to detect differences in BLL between first and third or second and third tertile, possibly due to a narrow range of BLL in the study sample. The difference in median energy intake between

second and third tertile did not differ significantly, and this could have also reduced our ability to detect differences between first and third or second and third tertile of these two macronutrients. In contrast, total energy or dietary protein intake (as energy %) was not associated with BLL in our study. Thus far, few studies have examined the relation between macronutrients and lead exposure in children (Elias et al 2007; Penuela et al 2006; Schell et al 2004; Gallichio et al 2002; Lucas et al 1996) or in adults (Park et al 2012; Arora et al 2008). The studies that did examine and association between carbohydrate intake and BLL in children did not find any significant relationships (Elias et al 2007; Lucas et al 1996), except for a study in infants that reported an inverse association between the intake of carbohydrate and children's BLL in unadjusted regression models (Gallichio et al 2002). In contrast, the finding of an inverse relation between fat intake and BLL in this study is consistent with other reports in children (Gallichio et al 2002; Lucas et al 1996). However, without the information on proportion of different types of fat (such as unsaturated or saturated) or carbohydrate (simple or complex) in the diet of our study children, it is not possible to comment on whether it was the intake a particular type of macronutrient or the overall intake that was associated with children's BLL. This should be investigated in future studies to better understand the role of macronutrients in lead exposure.

Among the micronutrient intakes tested in this study (calcium, iron, zinc, vitamin C and folate), dietary calcium intake was inversely associated with children's BLL in a dose-related manner. Although finding from this and other observational studies suggest that higher calcium intake may benefit children by lowering their BLLs, the supplementation of infants and preschoolers with calcium, has had limited success in in this respect (Keating et al 2011; Canfield et al 2005; Markowitz et al 2004; Sargent et al 1999). Therefore, caution should be taken in recommending calcium supplementation to reduce children's BLL before examining the

effects of calcium in well-designed randomized controlled studies in school-age or older children.

Finally, there was a weak positive association between BLL and the urinary concentrations of isoprostane in the study children, but no statistically significant association between children's BLL and the concentration of urinary 8-OH-dG. The interactive effects of antioxidants (vitamin C or zinc) with BLL on oxidative stress markers were not statistically significant. Isoprostanes are generated from the oxidation of tissue phospholipids (mostly arachidonic acid) by reactive oxygen species (ROS) and have been linked to chronic disease in adults (Kanaya et al 2011; Kaviarason et al 2009; Basu 2008). Lead may induce oxidative stress by several mechanisms, such as generating ROS, affecting antioxidant defense system, and altering the structure and function of cellular membranes (Ahamed & Siddiqui, 2007). There are few reports of a positive relation between BLL and lipid peroxidation in children (Cabral et al 2012, Ahamed et al 2011, 2008, 2006, and 2005). On the other hand, several experiments in animals and studies in occupationally-exposed adults suggest that exposure to high levels of lead is associated with higher lipid peroxidation (Haleagrahara et al 2011, Permpongpaiboon et al 2011, Khan et al 2008, Garcon et al 2007, Oktam et al 2004, Gurer-Orhan et al 2004). To our knowledge, ours was the first study to examine the relation between BLL and isoprostane or 8-OH-dG concentrations in children. Additionally, this was one of the few studies in children to test whether dietary intakes of vitamin C and zinc modify the relation between BLL and oxidative stress. Findings from this study suggest a potential adverse association between BLL and oxidative stress in children with low-level lead exposure and indicate a need to further study the effects of lead on other oxidative stress measures with a possibility for effect modification by antioxidants.

Accumulating evidence over the years suggests that low-level lead exposure is associated with adverse health outcomes. Yet, controversy still exists, particularly on the levels of lowest-observable-adverse-effects and some studies show that there is perhaps no safe threshold for lead exposure. Although, this study did not measure a health outcome, but examined oxidative stress, a process thought to be involved in many lead-related toxicities. Even a weak association between lead and one of the oxidative stress markers (marker for lipid peroxidation) in this study shows that lead even at a low level can be harmful and efforts to reduce lead exposure from the environment needs to be continued.

Altogether, this study provides preliminary information that could be useful in designing long-term studies examining the biological mechanisms behind lead toxicity and the role of nutritional factors to mitigate the exposure and toxicities of lead in children. For example, a prospective study could be designed where children can be followed longitudinally from infancy to adulthood. Information on lead exposure, oxidative stress parameters and nutritional status/intake could be collected to investigate the changes in exposure characteristics, the oxidative stress measures, to identify the risk factors for both oxidative stress and lead exposure and to examine the association between BLL and oxidative stress and whether this relationship changes. The role of nutrients or any components of the diet in modifying lead exposure and oxidative stress should also be examined. Apart from longitudinal studies, an intervention with educational (increasing awareness), behavioral (such as promoting better hygiene) and lifestyle components (such as reducing second-hand smoking, take-home exposure from workplace) could be designed targeting the parents and children to examine the effects of secondary prevention on children's BLL. Given the high cost of clinical trials or longitudinal studies, our findings could be beneficial for the design and implementation of these types of studies in the

future. The results from this study could be used to suggest or develop secondary strategies such as increasing awareness about environmental exposure, adopting behaviors and lifestyle to minimize exposure and a balanced diet that provide adequate nutrition to combat the problem of childhood lead exposure in Uruguay and elsewhere.

Study strengths and limitations

This study has several strengths. First, this study addresses some of the key issues regarding childhood lead exposure that are not well understood, but their better understanding can have the potential to inform preventive strategies against lead exposure and toxicity. Thus, the study addresses timely issues and important research gaps. Because abating lead from the environment is expensive and challenging, particularly for developing countries, finding cost-effective preventive strategies is important. Second, this was one of the few studies on lead exposure in Uruguayan children. This study provides evidence that low-level lead exposure is an environmental problem in school-age children in Montevideo. Since we collected data from several neighborhoods of Montevideo, this study presents further evidence that lead exposure is not restricted to one particular neighborhood of Montevideo but represents a more wide-spread problem, something that the society and the government will need to acknowledge. Furthermore, the socio-demographic and household factors identified in this study could be used to educate parents, inform health-care providers and policy makers.

Third, there are very few studies to examine the relationship between dietary intake of nutrients and BLL in school-age children. However, this is an important age-group to study both environmental exposure and dietary factors because exposure to environmental toxins and nutrient deficiencies can affect children's health, physical and cognitive growth and development

well into the school years. This, in turn, could potentially affect the health and productivity of these children as they reach adolescence and adulthood.

To our knowledge, this is the first study to examine isoprostane and 8-OH-dG concentrations as measures of oxidative stress in relation to BLL in children. However, these markers have been used to examine the relation between other toxins (such as arsenic and cadmium) and oxidative stress. Therefore, the result from this study could be the first step toward well-designed long-term mechanistic studies that would elucidate the role of oxidative stress in lead toxicity and the possible effect modification of lead-induced oxidative stress though nutritional or dietary strategies.

Finally, there are several methodological strengths of this study. First, we sampled children from several neighborhoods of Montevideo coming from low-to-middle income families, thus providing a good representation of vulnerable school-age children. Second, we were able to recruit more than 200 children and the drop-out rate in our study was quite low (~ 6%). Third, a detailed information on socio-demographic, family and child characteristics were collected. We also collected data on children's home environment. Third, the blood lead concentrations were analyzed by Atomic Absorption Spectrometry with high levels of quality control and sensitivity to detect low levels of lead. Moreover, standard procedure and quality control protocols were maintained for analyses of all the biochemical measurements including hemoglobin, serum CRP, ferritin, and urinary markers of oxidative stress. Detailed dietary data was collected over two days by five trained nutritionists using standard procedure and utmost care. There were no significant differences between the results of the recalls conducted by five different nutritionists. A widely used nutrient database that contained the nutrient composition of typical Uruguayan foods and preparations (altogether 342 unique items) were used to calculate

nutrient information. Furthermore, despite missing information on several socio-demographic variables, the use of a statistical technique (multiple imputation) to handle missing data made our findings in complete case analyses robust.

The findings of this study need to be considered in light of its limitations. First, this was a cross-sectional study, and therefore no cause-effect relationship can be established from its results. Second, the identification of socio-demographic and household predictors of children's BLL in this study was based on the self-reported parental questionnaire which could be subject to response bias. However, the identified factors predictive of BLL in these children are consistent with other studies and thus a response bias is less likely. Moreover, respondent-bias is more probable when the parents are aware of their child's BLL. Because the questionnaire was administered before the blood collection, parents in this study were unaware of their child's BLL when answering the questions. Still, it is possible that some of the responses such as the ones on family income, number of cigarettes smoked by parents per day or maternal smoking during pregnancy may be subject to some bias. We collected water, soil and dust from the homes of the participants. In the future, analyses of these environmental samples will help characterize lead exposure from these sources. Third, a limitation of this study was the dependence of the 24 hour recall on memory or the recall capability of the respondents. To reduce this, recalls were facilitated by probing about snacks and specific meals in details. In addition, food models and household measurement cups were used to facilitate food portion recalls. Nevertheless, recall is an issue in most dietary instruments, this problem is not inherent to the 24 hour recall. In addition, a food frequency questionnaire may be even more prone to issues of recall because people are asked to remember their intake over longer periods of time. The accuracy of dietary data also depends on the nutrient database used to calculate nutrient values. The nutrient

database used for this study is widely used in Uruguay, and had nutrient information of 342 unique food items, thus capturing an extensive range of Uruguayan foods. With lack of availability of dietary information in this population, our study is important in providing a snapshot of 5-8 year old children's diet. Furthermore, except for iron, this study lacks information on biomarkers of the nutrients queried during dietary recalls. This would have helped corroborate the information obtained on dietary intakes. However, because the plasma or serum levels of nutrients such as calcium and zinc are under tight physiological regulation, the available biomarkers are not very sensitive and specific to identify deficiencies of these micronutrients (Hambridge 2003; Potischman 2003). Therefore, information on intake of these nutrients is important to understand their status of in the body. Finally, this study is limited by a smaller sample size with missing data due to non-response, especially on socio-demographic variables. We used multiple imputation methods to handle the problem of missing data and our results with multiply imputed datasets were consistent with complete-case analyses. Nonetheless, our findings need to be tested in a larger sample in future studies.

Future directions

Low-level lead exposure still continues to threaten the health of children worldwide. Finding preventive strategies against this environmental problem is an important public health challenge. Effective dietary strategies and nutritional factors could prove to be a cost-effective preventive method. In the future, long-term studies should be conducted to follow children prospectively from infancy throughout childhood to better understand their exposure characteristics and the relation among diet, nutrients or any particular dietary component and children's BLL or its toxicities. Well-designed randomized controlled trials with particular nutrients (for example iron, zinc, calcium, vitamin C, and E) are required to establish cause-effect relation between a nutrient/s and BLL. However, previous supplementation studies to reduce BLL with micronutrient showed few benefits of supplementation. Nutrient supplementation was mostly effective in children with deficiency of the particular nutrient but not in children who already had adequate intake/status. Moreover, the efficacy of micronutrients in protecting against inhaled or lead absorbed through skin is questionable. Furthermore, the dose, timing and the duration of supplementation are important factors to consider. In addition to epidemiological investigations, mechanistic studies should be conducted to better understand the absorption and uptake of lead in the body, and the interaction with the nutrients at the absorptive and other tissues. An emphasis of the research on lead should also be the understanding of the molecular mechanisms behind lead toxicity, including investigating the role of oxidative stress as a potential molecular mechanism for lead-induced toxicities. Future studies in this field should focus on more detailed investigation of oxidative stress measures, including the status of

antioxidant enzymes, non-enzymatic antioxidants, along with lipid peroxidation, DNA and protein damage products. This will provide a comprehensive understanding of the nature and extent of the molecular damage caused by lead. Moreover, studies should examine whether oxidative stress is a potential mediator in the relation between lead exposure and adverse biochemical consequences such as inflammation or functional outcomes such as neurodevelopmental problems, hypertension and diabetes. Furthermore, the role of dietary intake of antioxidant nutrients such as vitamin A, E, C, selenium, and zinc, along with specific food groups, in protecting against lead-induced oxidative stress should be investigated. Finally, designing environmental education interventions, especially for the children in Uruguay and other developing countries, is important because there is a lack of data on the effectiveness of such programs in reducing lead exposure in children.

CONCLUSION

In Uruguayan first-grade children there was evidence of continued lead exposure even after the removal of lead from gasoline. Children's blood lead values were at a level shown in many studies to be associated with cognitive and behavioral deficits. This in turn could affect children's academic performance and school achievement. There is a need for blood lead monitoring and prevention of exposure in this population. This study identified most salient socio-demographic and household predictors of blood lead levels in these school-age children. These predictors included father's smoking, father's job exposure risk and having more than one young child at home. Among the dietary nutrients associated with BLL were carbohydrate, fat and calcium intake. Dietary intake of carbohydrate or fat was positively associated with BLL, but no dose-response relation was found. On the other hand, dietary calcium intake was inversely associated with BLL in a dose-related manner. Intakes of total energy, protein, and micronutrients such as iron, zinc, vitamin C or folate were not associated with BLL in the study children. Furthermore, the interactions between pairs of micronutrients, such as iron-zinc, iron-calcium and iron-vitamin C, did not modify children's BLLs. When the relationship between BLL and oxidative stress was tested to better understand the biological mechanism behind lead toxicity, there was a weak association between higher BLL and higher concentration of urinary F_2-α isoprostane (isoprostane), but no association with urinary concentration of 8-hydroxy- 2-deoxy guanosine (8-OH-dG), suggesting that even at low levels, lead begins to induce damage at a molecular level in humans. Continued exposure to low-level lead could contribute to chronic disease states. No significant interaction effects between BLL and vitamin C or zinc were found

in the study children, possibly suggesting that at this level of exposure antioxidants are not helpful in reducing the molecular damage, but this needs to be confirmed with additional studies.

Given the extent of lead exposure in this population, lead testing should be made a routine practice as a part of pediatric care. In addition, some of the factors/behaviors identified in this study are modifiable and can be target of intervention to reduce lead exposure in these children. Health-care providers, public-health specialists, educators and the government could initiate efforts such as promoting a smoke-free environment in the home, increasing awareness at the work-place to reduce take-home exposure to lead and other metals, educating parents, teachers and children to change behaviors and life-style. Educational campaigns around the reduction of risk factors for environmental exposure could be useful if initiated at the school. The findings of this study on dietary nutrients and children's BLL should be tested in longitudinal and well-designed intervention studies to formulate a comprehensive dietary strategy for the reduction of BLL and lead toxicity. To confirm the results of this study regarding BLL and oxidative stress, future studies should include larger samples and additional measures of oxidative stress. It is also important to examine whether oxidative stress is a potential mediator in the relation between lead exposure and adverse biochemical consequences (for example inflammation) or functional outcomes (such as neurodevelopmental problems, hypertension and diabetes). Finally, the role of dietary intake of antioxidant nutrients along with specific food groups or dietary components in protecting against lead-induced oxidative stress should be investigated in the future.

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