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Integrated Science Assessment for Lead

Appendix 7: Hematological Effects

External Review Draft

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Health and Environmental Effects Assessment Division
Center for Public Health and Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency

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DOCUMENT GUIDE

This Document Guide is intended to orient readers to the organization of the Lead (Pb) Integrated Science Assessment (ISA) in its entirety and to the sub-section of the ISA at hand (indicated in bold). The ISA consists of the Front Matter (list of authors, contributors, reviewers, and acronyms), Executive Summary, Integrated Synthesis, and 12 appendices, which can all be found at https://cfpub.epa.gov/ncea/isa/recordisplay.cfm?deid=357282.

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ACRONYMS AND ABBREVIATIONS

ALAD	δ-aminolevulinate dehydratase
ALA	aminolevulinic acid
AQCD	Air Quality Criteria Document
BLL	blood lead level
BMI	body mass index
BW	body weight
Ca^{2+}	calcium
CAT	catalase
CI	confidence interval
e-waste	electronic waste
EPA	Environmental Protection Agency
EPO	erythropoietin
Fe^{2+}	iron ion
GFAAS	graphite furnace atomic absorption spectroscopy
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
Hb	hemoglobin
Hct	hematocrit
HSC	hematopoietic stem cell
OH	hydroxyl radical
h	hour, hours
H2O2	hydrogen peroxide
ICP-MS	inductively coupled plasma mass spectrometry
KNHANES	Korean National Health and Nutrition Examination Survey
ln	natural logarithm
M	male
M/F	male/female
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MDA	malondialdehyde
Mg^{2+}	magnesium ion
mo	month, months
NAAQS	National Ambient Air Quality Standards
NCE	normochromatic red blood cell
NR	not reported
OR	odds ratio
Pb	lead
PCE	polychromatic red blood cells
NCE	normochromatic red blood cells
PCV	packed cell volume
PECOS	Population, Exposure, Comparison, Outcome, and Study Design
Plt	platelets
PND	postnatal day
PS	phosphatidylserine
Q	quartile
RBC	red blood cell
RDW	red blood cell distribution width
ROS	reactive oxygen species
SD	Sprague Dawley
SES	socioeconomic status
SOD	superoxide dismutase
O_2-	superoxide
wk	week, weeks
yr	year, years
Zn	zinc
ZPP	zinc-protoporphyrin

APPENDIX 7 HEMATOLOGICAL EFFECTS

Causality Determination for Pb Exposure and Hematological Effects

This appendix characterizes the scientific evidence that supports the causality determination for lead (Pb) exposure and hematological effects. The types of studies evaluated within this appendix are consistent with the overall scope of the ISA as detailed in the Process Appendix (see Section 12.4). In assessing the overall evidence, the strengths and limitations of individual studies were evaluated based on scientific considerations detailed in Table 12-5 of the Process Appendix (Section 12.6.1). More details on the causal framework used to reach these conclusions are included in the Preamble to the ISA (U.S. EPA 2015). The evidence presented throughout this appendix supports the following causality determination:

Outcome	Causality Determination
Altered Heme Synthesis and Decreased Red Blood Cell Survival and Function	Causal

The Executive Summary, Integrated Synthesis, and all other appendices of this Pb ISA can be found at https://cfpub.epa.gov/ncea/isa/recordisplay.cfm?deid=357282.

7.1 Introduction, Summary of the 2013 Integrated Science Assessment, and Scope of the Current Review

Hematology is a subgroup of clinical pathology concerned with the morphology, physiology, and pathology of blood and blood-forming tissues. Hematological measures, when evaluated with information on other biomarkers, are informative diagnostic tests for blood-forming tissues (i.e., bone marrow, spleen, and liver) and organ function.

The 2013 Integrated Science Assessment for Lead (hereinafter referred to as the 2013 Pb ISA) issued causality determinations for hematological effects resulting from lead (Pb) exposure on red blood cell (RBC) survival and function and altered heme synthesis (U.S. EPA 2013). The evidence underpinning these causality determinations is summarized below. Given the interconnectedness of the effects of Pb on RBC survival and function and altered heme synthesis, this assessment presents a single causality determination for Pb exposure and hematological effects. This approach allows for a more holistic evaluation of inter-related health endpoints, including a discussion of how all individual lines of evidence contribute to the overall hematological effects causality determination.

7.1.1. Red Blood Cell Survival and Function

The body of epidemiologic and toxicological evidence assessed in the 2013 Pb ISA indicates a "causal" relationship between Pb exposure and decreased RBC survival and function. Experimental animal studies demonstrate that relevant human blood Pb levels (BLLs) from oral and inhalation exposure alter several hematological parameters (e.g., RBC number), increase measures of oxidative stress (e.g., inhibition of antioxidant enzymes in RBCs), and increase cytotoxicity in RBC precursor cells. Some of these effects have been observed in animal toxicological studies with exposures resulting in BLLs of 2–7 μg/dL. Evidence of biologically plausible modes of action, including increased intracellular calcium concentrations $[Ca^{2+}]_i$, decreased Ca^{2+}/Mg^{2+} ATPase activity, and increased phosphatidylserine (PS) exposure leading to RBC destruction by macrophages, support these findings. Epidemiologic studies reported associations between exposure to Pb, BLL, and altered hematological endpoints, increased measures of oxidative stress, and altered hematopoiesis in adults and children. Although most of these studies are limited by their lack of rigorous methodology (i.e., correlations, t tests, or chi squared analyses), some studies in children did adjust for potential confounding factors, including age, sex, mouthing behavior, anemia, dairy product consumption, maternal age, education, employment, marital status, family structure, and socioeconomic status (SES)-related variables. Though limited in number, studies that adjusted for confounders also reported consistent associations between BLL and altered hematological parameters, strengthening their support for findings in experimental animals. Collectively, the strong evidence from toxicological studies supported by findings from mode of action and epidemiologic studies reviewed in the 2013 Pb ISA was sufficient to conclude that there is a causal relationship between Pb exposures and decreased RBC survival and function.

7.1.2. Heme Synthesis

Available toxicological evidence evaluated in the 2013 Pb ISA indicated a causal relationship between Pb exposure and altered heme synthesis (U.S. EPA 2013). Altered heme synthesis is demonstrated by a small but consistent body of studies in adult animals, which report that exposures that result in BLLs relevant to humans (e.g., 10 μg/dL) lead to decreased δ-aminolevulinate dehydratase (ALAD) and ferrochelatase activities. Supporting this evidence is a larger body of ecotoxicological studies that demonstrate decreased ALAD activity across a wide range of taxa exposed to Pb. Evidence of biologically plausible modes of action, including evidence that Pb acts directly on two enzymes involved in heme synthesis (ALAD and ferrochelatase), decreased RBC Hb concentration, measures of oxidative stress, and evidence that administration of antioxidants reduced the effects of Pb exposure on antioxidant enzymes, support these findings. Epidemiologic studies find associations in both adults and children between higher BLLs and decreased ALAD and ferrochelatase activities. Although most of these studies are limited by their lack of rigorous methodology and consideration of potential confounders, some studies in children did incorporate potential confounding factors (i.e., age, sex, urban/rural residence,

height, weight, BMI [body mass index]). Although limited in number, studies that adjusted for confounders also reported consistent associations between BLLs and decreased ALAD and ferrochelatase, strengthening support for the findings in the animal toxicological studies. Evidence for altered heme synthesis is also provided by a large body of toxicological and epidemiologic studies that report decreased hemoglobin (Hb) concentrations in association with Pb exposure or BLL. The 2013 Pb ISA concluded that, collectively, the strong evidence from toxicological and ecotoxicological studies—supported by findings from epidemiologic studies—is sufficient to conclude a causal relationship between Pb exposures and altered heme synthesis.

This ISA determined causality for adverse effects of Pb exposure on altered heme Synthesis and decreased red blood cell survival and function. Recent evidence demonstrates that Pb exposures alter several hematological parameters, decrease enzyme activity related to heme synthesis, and increase RBC oxidative stress. Biological plausibility is provided by toxicological and epidemiologic studies demonstrating increased intracellular calcium concentrations, decreased Ca^{2+}/Mg^{2+} ATPase activity, and increased PS exposure. Taken together, there is sufficient evidence to conclude that there is a causal relationship between Pb exposure and hematological effects, including altered heme synthesis and decreased RBC survival and function.

The following sections provide an overview of the scope of the appendix (Section 7.2), evaluation of the scientific evidence relating Pb exposures and hematological effects (Sections 7.3 and 7.4), a discussion of biological plausibility (Section 7.5), and a summary section with the updated causality determination (Section 7.6, Table 7-1). The focus of these sections is on studies published since the completion of the 2013 Pb ISA (U.S. EPA 2013). Study-specific details, including animal type, exposure concentrations and exposure durations in experimental studies, and study design; exposure metrics; and select results in epidemiologic studies are presented in evidence inventories in Section 7.7.

7.2 Scope

The scope of this section is defined by Population, Exposure, Comparison, Outcome, and Study Design (PECOS) statements. The PECOS statement defines the objectives of the review and establishes study inclusion criteria, thereby facilitating identification of the most relevant literature to inform the Pb ISA.[1] To identify the most relevant literature, the body of evidence from the 2013 Pb ISA was considered in the development of the PECOS statements for this appendix. Specifically, well-established areas of research; gaps in the literature; and inherent uncertainties in specific populations, exposure metrics,

[1] The following types of publications are generally considered to fall outside the scope and are not included in the ISA: review articles (which typically present summaries or interpretations of existing studies rather than bringing forward new information in the form of original research or new analyses), Pb poisoning studies or clinical reports (e.g., involving accidental exposures to very high amounts of Pb described in clinical reports that may be extremely unlikely to be experienced under ambient air exposure conditions), and risk or benefits analyses (e.g., that apply concentration-response functions or effect estimates to exposure estimates for differing cases).

comparison groups, and study designs identified in the 2013 Pb ISA inform the scope of this appendix. The 2013 Pb ISA used different inclusion criteria than the current ISA, and the studies referenced therein often do not meet the current PECOS criteria (e.g., due to higher or unreported biomarker levels). Studies that were included in the 2013 Pb ISA, including many that do not meet the current PECOS criteria, are discussed in this appendix to establish the state of the evidence prior to this assessment. With the exception of supporting evidence used to demonstrate the biological plausibility of Pb-associated hematological effects, recent studies were only included if they satisfied all of the components of the following discipline-specific PECOS statements:

Epidemiologic Studies

Population: Any human population, including specific populations or lifestages that might be at increased risk of a health effect;
Exposure: Exposure to Pb[1] as indicated by biological measurements of Pb in the body, with a specific focus on Pb in blood, bone, and teeth; validated environmental indicators of Pb exposure[2]; or intervention groups in randomized trials and quasi-experimental studies;
Comparison: Populations, population subgroups, or individuals with relatively higher versus lower levels of the exposure metric (e.g., per unit or log unit increase in the exposure metric, or categorical comparisons between different exposure metric quantiles);
Outcome: Hematological effects including but not limited to disruption of heme synthesis and RBC survival and function; and
Study Design: Epidemiologic studies consisting of longitudinal and retrospective cohort studies, case-control studies, cross-sectional studies with appropriate timing of exposure for the health endpoint of interest, randomized trials, and quasi-experimental studies examining interventions to reduce exposures.

Experimental Studies

Population: Laboratory nonhuman mammalian animal species (e.g., mouse, rat, guinea pig, minipig, rabbit, cat, dog) of any lifestage (including preconception, in utero, lactation, peripubertal, and adult stages);
Exposure: Oral, inhalation or intravenous treatment(s) administered to a whole animal (in vivo) that results in a BLL of 30 mg/dL or below;[3,4]
Comparators: A concurrent control group exposed to vehicle-only treatment or untreated control;

[1] Recent studies of occupational exposure to Pb were only considered insofar as they addressed a topic area that was relevant to the National Ambient Air Quality Standards review (e.g., longitudinal studies designed to examine recent versus historical Pb exposure).
[2] Studies that estimate Pb exposure by measuring Pb concentrations in particulate matter with a nominal mean aerodynamic diameter less than or equal to 10 μm^3 (PM_{10}) and particulate matter with a nominal mean aerodynamic diameter less than or equal to 2.5 μm^3 ($PM_{2.5}$) ambient air samples are only considered for inclusion if they also include a relevant biomarker of exposure. Given that size distribution data for Pb-PM are limited, it is difficult to assess the representativeness of these concentrations to population exposure [Section 2.5.3 (U.S. EPA 2013)]. Moreover, data illustrating the relationships of Pb-PM_{10} and Pb-$PM_{2.5}$ with BLLs are lacking.
[3] Pb mixture studies are included if they employ an experimental arm that involves exposure to Pb alone.
[4] This level is approximately an order of magnitude above the upper end of the distribution of U.S. young children's BLLs. The 95th percentile of the 2011–2016 National Health and Nutrition Examination Survey distribution of BLL in children (1–5 years; n = 2,321) is 2.66 µg/dL (CDC 2019) and the proportion of individuals with BLL that exceed this concentration varies depending on factors including (but not limited to) housing age, geographic region, and a child's age, sex and nutritional status.

Outcome: Hematological effects; and
Study design: Controlled exposure studies of animals in vivo.

7.3 Red Blood Cell Survival and Function

Toxicological and epidemiologic studies evaluated in the 2013 Pb ISA (U.S. EPA 2013) provided strong evidence that exposure to Pb affects a range of hematological outcomes related to RBC survival and function; which is consistent with epidemiologic evidence from the 2006 Pb Air Quality Criteria Document (AQCD) (U.S. EPA 2006b), demonstrating an association between high BLLs and anemia in children. Given the extensive evidence base at higher BLLs, the scope for this appendix focuses on toxicological studies conducted at lower exposure levels and epidemiologic studies in nonoccupational populations (as described in Section 7.2). Under the defined PECOS criteria, recent toxicological and epidemiologic studies provide additional support for Pb-related changes in Hb concentration and some other hematological measures of RBC survival and function. Below, recent evidence is reviewed in the context of evidence from past assessments.

7.3.1. Epidemiologic Studies of Red Blood Cell Survival and Function

The epidemiologic evidence evaluated in the 2013 Pb ISA (U.S. EPA 2013) covered a range of measures related to RBC survival and function, including RBC counts and other hematological parameters, hematopoiesis, Ca^{2+}/Mg^{2+} ATPase activity, PS exposure, and RBC oxidative stress. Specifically, epidemiologic studies provided evidence that elevated BLLs in children and adults are associated with altered hematological parameters (e.g., decreased RBC counts, Hb concentration, and hematocrit [Hct] and changes in mean corpuscular volume [MCV] and mean corpuscular hemoglobin [MCH]), increased measures of oxidative stress (e.g., altered antioxidant enzyme activities [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)], decreased cellular glutathione (GSH), and increased lipid peroxidation), and altered hematopoiesis (e.g., decreased erythropoietin [EPO]). Notably, most of these epidemiologic studies are cross-sectional in design and conducted either in occupationally exposed populations or other populations with higher mean Pb exposures (i.e., BLLs >10 μg/dL). These studies were additionally limited by their lack of consideration of potential confounders, although some studies in children did adjust for a range of factors, including age, sex, mouthing behavior, anemia, dairy product consumption, maternal age, education, employment, marital status, family structure, and SES-related variables (Queirolo et al. 2010; Ahamed et al. 2007; Riddell et al. 2007). Studies that did account for potential confounders reported consistent associations between increased BLLs and decreased Hb levels, increased prevalence of anemia, and increased RBC oxidative stress (Sections 4.7.2.1 and 4.7.2.7 of the 2013 Pb ISA). As a whole, the cross-sectional study designs, higher exposures, and lack of rigorous statistical methodologies in many studies raise uncertainties in the epidemiologic evidence regarding the directionality of effects; the level, timing, frequency, and duration

of Pb exposure that contributed to the observed associations; and whether the observed associations are independent of potential confounders.

Recent epidemiologic studies provide generally consistent evidence of associations between Pb exposures and decreased Hb levels in children. These associations are reported at lower BLLs than in studies included in the 2013 Pb ISA. Evidence for associations with other hematological parameters of RBC function and survival, as well as Hb levels in adults, is less robust. Consistent with the 2013 Pb ISA, most recent studies are cross-sectional analyses, which are unable to establish temporality between exposure and outcome. However, recent studies include populations with lower mean BLLs and more robust adjustment for potential confounders compared with studies included in the 2013 Pb ISA. Measures of central tendency for BLLs used in each study, along with other study-specific details including study population characteristics and select effect estimates, are highlighted in Table 7-2. An overview of the recent evidence is provided below.

The most common hematological parameter evaluated in recent epidemiologic studies is Hb levels. A number of cross-sectional studies of children in China observed decreases in Hb levels associated with increases in blood Pb or erythrocyte Pb levels (Guo et al. 2021; Kuang et al. 2020; Li et al. 2018; Liu et al. 2015; Liu et al. 2012). Kuang et al. (2020) and Li et al. (2018) also reported inverse associations between BLLs and MCH in children, which is a measure of average Hb concentration in a single erythrocyte. Notably, all of these studies had mean and/or median BLLs below 10 µg/dL, including some below 5 µg/dL (Guo et al. 2021; Kuang et al. 2020; Liu et al. 2012). While only a few studies attempted to account for potential confounding by SES (Kuang et al. 2020; Liu et al. 2015), others adjusted directly for iron deficiency (Li et al. 2018; Liu et al. 2012), which may be the direct mechanism by which SES could potentially confound the relationship between BLLs and Hb (i.e., via nutritional deficiency). Although the magnitude of the observed effect estimates was not directly comparable across studies, BLLs were negatively associated with Hb levels in all studies. Notably, Liu et al. (2012) reported that a 1 µg/dL increase in BLLs was associated with a larger decrease in Hb levels when restricting their sample to children with BLLs less than 10 µg/dL (−0.174 g/dL [95% confidence interval (CI): −0.27, −0.078 g/dL]) compared with the full sample (−0.096 g/dL [95% CI: −0.18, −0.012 g/dL]). In studies that examined quantiles of exposure, blood or erythrocyte Pb levels were only associated with Hb levels at the higher quantiles (Guo et al. 2021; Liu et al. 2015). For example, in a large hospital-based study in China, children with BLLs between 3.33 and 4.50 µg/dL (quintile 4) and those with levels greater than 4.50 µg/dL (quintile 5) had decreases in Hb relative to children with BLLs less than 1.61 µg/dL (−0.49 g/L [95% CI: −0.94, −0.04 g/L] and −1.25 g/L [95% CI: −1.71, −0.78 g/L], respectively); however, null associations were reported for quintiles 2 (1.61–2.44 µg/dL) and 3 (2.44–3.33 µg/dL) relative to the lowest quintile of exposure (Guo et al. 2021). While the clinical relevance of small mean decrements in Hb across exposure quintiles is unclear, Guo et al. (2021) also reported monotonic increases in the odds of anemia (defined as Hb levels below 110 g/L) in association with increasing blood Pb quintiles, with a 45% (95% CI: 26%, 67%) increase in the odds of anemia for children in the highest

quintile of exposure relative to the lowest quintile. Similarly, Li et al. (2018) observed a 5% (95% CI: 0%, 11%) increase in the odds of decreased Hb levels (<115 g/L) per 1 µg/dL increase in blood Pb.

Recent studies of Pb exposure and Hb levels in adults are more limited in number and include overlapping study populations. In contrast to studies in children, Park and Lee (2013) reported increases in Hb associated with continuous increases in BLLs for adult participants of the 2008–2010 cycles of the Korean National Health and Nutrition Examination Survey (KNHANES). The analysis was stratified by sex and the observed associations were comparable for men and women. A similar study analyzed the same KNHANES cycles, but examined the population as a whole, rather than stratified by sex, and also categorized exposure into quartiles (Kim and Lee 2013). The authors noted similar positive associations between blood Pb and Hb levels. However, Kim and Lee (2013) observed inverse associations across exposure quartiles when correcting BLLs for Hct in order to estimate erythrocyte Pb. As described in the 2006 Pb AQCD (U.S. EPA 2006b), Pb exposure decreases Hct and MCV, meaning the negative effects of Pb can potentially decrease Pb levels in whole blood.

In addition to studies examining the relationship between Pb exposure and Hb levels, a few recent cross-sectional studies evaluate associations between BLLs and other hematological parameters. In a group of children in China, including some living near a battery plant or a lead/-zinc mine, Li et al. (2018) reported increased odds of low RBC counts and low blood platelets (Plt) in association with increased BLLs. In contrast, Kuang et al. (2020) observed a positive association with increased RBC counts in a convenience sample of slightly older boys in Nanjing, China, with notably lower median BLLs (2.61 µg/dL compared with 8.38 µg/dL). In an adult population, a cohort of pregnant women in Durango, Mexico, La-Llave-León et al. (2015) also observed a positive cross-sectional association between RBC counts and BLLs. Given this small body of studies that examine diverse populations with varying BLLs, it is difficult to discern methodological or demographic factors contributing to the inconsistent results.

7.3.2. Toxicological Studies of Red Blood Cell Survival and Function

As previously reported in the 2013 Pb ISA, epidemiologic evidence is coherent with experimental animal studies demonstrating that exposures via drinking water and oral gavage resulting in BLLs relevant to what was found in humans affect multiple hematological outcomes related to RBC survival and function (U.S. EPA 2013). Specifically, exposure to Pb has been shown to decrease RBC survival, either through direct effects on RBC membranes leading to increased fragility or through the induction of eryptosis and eventual phagocytosis by macrophages (U.S. EPA 2013). Some of these effects have been observed in animal toxicological studies with exposures resulting in 2–7 µg/dL BLL. For example, Hb concentrations in plasma was significantly decreased in male mice exposed to Pb nitrate (50 mg/kg BW in drinking water for 40 days; BLL: 1.72 ± 0.02 µg/dL) (Sharma et al. 2010). BLLs >100 µg/dL were also associated with decreased RBC survival in laboratory animals.

The evidence is limited and conflicting for the observed effects of Pb exposure on hematopoiesis in rats and mice. For example, administration of Pb acetate (140, 250, or 500 mg/kg) via oral gavage once per week for 10 weeks decreased the number of polychromatic RBCs (PCE) and increased numbers of micronucleated PCEs in female rats (Çelik et al. 2005). Increased micronucleated PCEs were reported in female and male rats exposed to Pb acetate in drinking water for 125 days, but decreased PCEs/normochromatic RBCs (NCEs) ratio was only observed in male rats (Alghazal et al. 2008). However, in mice exposed to Pb acetate (1 g/L in drinking water for 90 days), PCE increased, but PCE/NCE was unaffected (Marques et al. 2006).

In addition, the 2013 Pb ISA reported that Pb exposure significantly decreases several hematological parameters. In studies reporting BLL relevant to this ISA, decreased RBC counts (Andjelkovic et al. 2019; Cai et al. 2018; Sharma et al. 2010), Hb concentration (Andjelkovic et al. 2019; Cai et al. 2018; Berrahal et al. 2011; Sharma et al. 2010; Baranowska-Bosiacka et al. 2009; Massó-González and Antonio-García 2009), and Hct (Andjelkovic et al. 2019; Massó-González and Antonio-García 2009; Massó et al. 2007) were reported in laboratory studies conducted in rats. In other studies in which BLLs were not reported, decreased RBC counts (Simsek et al. 2009; Marques et al. 2006; Lee et al. 2005), Hb (Wang et al. 2010b; Simsek et al. 2009; Lee et al. 2005), Hct (Molina et al. 2011; Marques et al. 2006; Lee et al. 2005), MCV (Wang et al. 2010b), MCH (Wang et al. 2010b; Simsek et al. 2009), and mean corpuscular hemoglobin concentration (MCHC) (Wang et al. 2010b; Simsek et al. 2009) were reported in laboratory studies conducted in rats and mice. Some toxicological studies found no evidence of hematological effects (Gautam and Flora 2010; Lee et al. 2006).

Recent studies also report effects of Pb exposure on hematological parameters at BLLs relevant to this ISA. Administration of Pb acetate in drinking water (0.2%; BLL = 9.3 ± 0.98 µg/dL) for 84 days resulted in RBC hemolysis, and significantly decreased RBC lifespan and number, and Hb levels, but had no effect on Plt number in blood collected from Sprague Dawley rats (Cai et al. 2018). In a different study, administration of Pb acetate in drinking water (0.150 mg/kg; BLL = 14.7 µg/dL) for 1 day decreased RBCs, Plts, Hb concentration, and Hct, but had no effect on MCV, MCH an MCHC in whole blood collected from adult male Wistar rats (Andjelkovic et al. 2019). Pb acetate treatment increased Hct and RBC distribution width, decreased MCHC, and had no effect on RBC number, Hb, MCV, and MCH in adult male C57BJ mice exposed via drinking water (200 ppm; BLL = 21.6 µg/dL) for 45 days (Corsetti et al. 2017). The potential effects of Pb exposure in rats were also investigated through a combination of lactational and drinking water exposures. Beginning on postnatal day (PND) 1, dams were given drinking water containing Pb acetate (50 mg/L). On PND 21, male pups were subsequently administered Pb acetate (50 mg/L) in drinking water for an additional 40 or 65 days, at which time they were sacrificed. Hct was significantly reduced in mice exposed until PND 40 (BLL = 12.67 ± 1.68 µg/dL) whereas Hct and Hb were significantly decreased at PND 65 (BLL = 7.49 ± 0.78 ug/dL) (Berrahal et al. 2011). Study-specific details, including animal species, strain, sex, and BLLs are highlighted in Table 7-3.

7.3.3. Integrated Summary of Red Blood Cell Survival and Function

Experimental animal studies evaluated in the 2013 Pb ISA (U.S. EPA 2013) demonstrate that Pb exposures resulting in BLLs relevant to humans (i.e., <10 µg/dL) alter several hematological parameters, increase measures of oxidative stress, and increase cytotoxicity in RBC precursor cells. While epidemiologic evidence synthesized in the last review was generally coherent with results from the animal studies, most of the epidemiologic studies evaluated are cross-sectional, were conducted in populations with higher mean Pb exposures (i.e., BLLs >10 µg/dL), did not thoroughly consider potential confounders, and lacked rigorous statistical methodology. As a result, there were considerable uncertainties in the epidemiologic evidence regarding the directionality of effects; the level, timing, frequency, and duration of Pb exposure that contributed to the observed associations; and whether the observed associations are independent of potential confounders.

Though limited in number, recent PECOS-relevant animal toxicological studies continue to support the findings from the last review. Specifically, these studies consistently report the effects of Pb on hematological parameters, including mostly consistent evidence of a Pb-related decrease in Hb. Recent epidemiologic studies expand on the evidence presented in the 2013 Pb ISA and provide additional support for the experimental evidence. Although the recent studies are also cross-sectional, they include populations with much lower BLL means (<10 µg/dL) and include more robust adjustment for potential confounding, addressing important uncertainties from the last review. The most consistent epidemiologic evidence indicates an association between BLLs and decreased Hb levels in children, which is coherent with the evidence from recent experimental animal studies. While the clinical relevance of small mean decrements in Hb is unclear, a few of the recent epidemiologic studies include analyses linking increased BLLs to increased prevalence of anemia. Recent epidemiologic studies of Hb levels in adults were more limited in number and less consistent than those in children. Additionally, of the relatively few studies examining RBC counts, the results were also inconsistent.

7.4 Heme Synthesis

Toxicological and ecotoxicological studies evaluated in the 2006 Pb AQCD (U.S. EPA 2006b) and the 2013 Pb ISA (U.S. EPA 2013) provided strong evidence that exposure to Pb affects heme synthesis. A limited number of epidemiologic studies contributed compelling supporting evidence. Given the extensive evidence base at higher BLLs, the scope for this appendix focuses on toxicological studies conducted at lower exposure levels and epidemiologic studies in nonoccupational populations (as described in Section 7.2). Below, recent evidence is reviewed in the context of evidence from past assessments.

7.4.1. Epidemiologic Studies of Heme Synthesis

The epidemiologic studies evaluated in the 2013 Pb ISA (U.S. EPA 2013) provided evidence that BLLs in children and adults are associated with decreased activity of enzymes involved in the heme synthesis pathway, including ALAD and ferrochelatase. Similar to studies of RBC function and survival, most studies on heme synthesis are cross-sectional in design and conducted either in occupationally exposed populations and/or in populations with higher mean Pb exposures (i.e., BLL >20 µg/dL). These studies were additionally limited by their lack of consideration of potential confounders, although some studies adjusted for or consider potential confounding factors (i.e., age, sex, urban/rural residence, height, weight, BMI (Wang et al. 2010a; Ahamed et al. 2007; Ahamed et al. 2006)). Studies that did account for potential confounders reported consistent associations between increased BLLs and decreased ALAD activity (Section 4.7.3.1 of the 2013 Pb ISA). Evidence for altered heme synthesis is also provided by the epidemiologic studies discussed in Section 7.3.1 that report decreased Hb concentrations in association with increased Pb exposure or BLLs. Decreased RBC survival and hematopoiesis can be expected to occur simultaneously, and any effect on Hb levels is likely a combination of the two processes. Outside of the recent studies on Hb concentrations discussed in Section 7.3.1, there are no recent PECOS-relevant epidemiologic studies examining the relationship between Pb exposure and heme synthesis.

7.4.2. Toxicological Studies of Heme Synthesis

Pb-induced alterations in heme synthesis occurring at BLLs relevant to this ISA (e.g., 10 µg/dL) have been demonstrated convincingly by a small but consistent body of evidence. In brief, Pb exposure in rats inhibits several enzymes involved in heme synthesis, most notably ALAD, the enzyme that catalyzes the second, rate-limiting step in heme biosynthesis (Rendón-Ramirez et al. 2007; Terayama et al. 1986). Pb exposure has also been shown to inhibit ferrochelatase, a mitochondrial iron (Fe)-sulfur (S) containing enzyme that incorporates Fe^{2+} into protoporphyrin IX to create heme (Rendón-Ramirez et al. 2007).

Toxicological studies have found that Pb exposures result in increases in markers of oxidative stress. For example, Lee et al. (2005) reported increased RBC malondialdehyde (MDA), SOD and CAT levels accompanied by significant decreases in GSH and GPx in rats exposed to Pb (25 mg/kg) once a week for 4 weeks. In a second drinking water study performed in rats, administration of Pb acetate (750 mg/kg in drinking water for 11 weeks) resulted in decreased concentrations of plasma Vitamin C, Vitamin E, nonprotein thiol, and RBC-GSH, with simultaneous increased activity of SOD and GPx (Kharoubi et al. 2008). Effects on measures of oxidative stress were also observed in in vitro studies including increased MDA and decreased SOD and CAT in RBCs (Ciubar et al. 2007), and decreased glutathione reductase (GR) activity in human RBCs (Coban et al. 2007), and decreased GSH and increased glutathione disulfide (GSSG).

There were no recent toxicology studies investigating the effects of Pb exposure on heme synthesis that satisfied the PECOS criteria described in Section 7.2 available for this review.

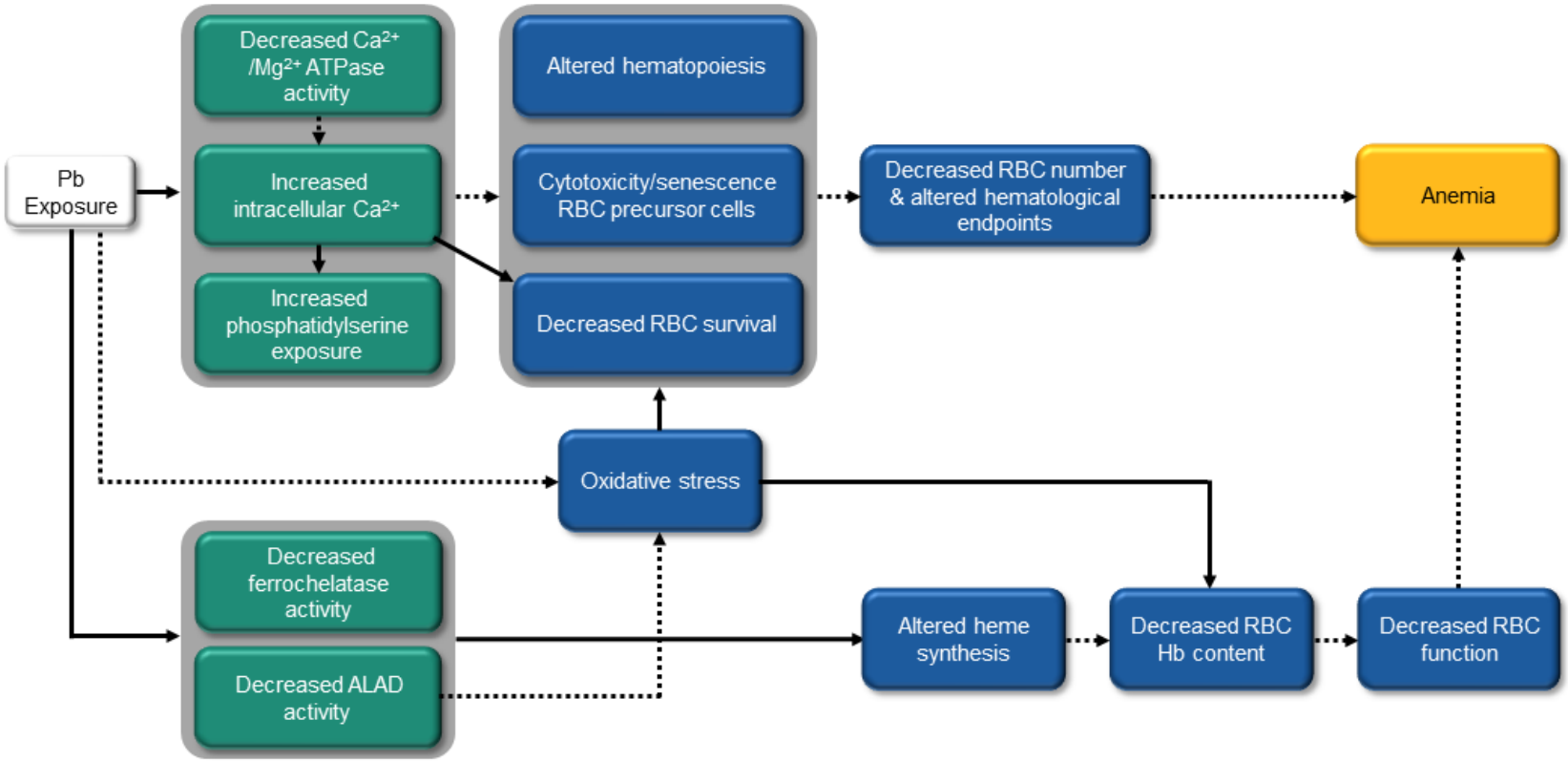
7.4.3. Integrated Summary of Heme Synthesis

A small number of animal toxicological studies evaluated in the 2013 Pb ISA provide consistent evidence that Pb exposures affect heme synthesis, including Pb-induced decreases in ALAD (Rendón-Ramirez et al. 2007; Terayama et al. 1986) and ferrochelatase activities (Rendón-Ramirez et al. 2007). The toxicological evidence was supported by a larger body of ecotoxicological studies that demonstrate ALAD inhibition in Pb-exposed aquatic and terrestrial invertebrates and vertebrates (Sections 6.3.4.3, 6.4.5.2, 6.4.5.3, and 6.4.15.2 of the 2013 Pb ISA). Ecological evidence from previous reviews consistently observed Pb-induced ALAD inhibition in multiple species, including birds and fish (U.S. EPA 2013, 2006b). Some cross-sectional epidemiologic studies evaluated in previous reviews provide supporting evidence that concurrent BLLs are associated with decreased ALAD and ferrochelatase activities in both adults and children. The majority of these studies, however, are limited by the lack of rigorous methodology and consideration of potential confounding.

Recent evidence provided by epidemiologic and toxicological studies of Pb exposure and Hb levels provides additional support for Pb-related impairment of heme synthesis. These studies are discussed in more detail in Section 7.3.

7.5 Biological Plausibility

This section describes biological pathways that potentially underlie effects on hematology measures resulting from exposure to Pb. Figure 7-1 depicts the proposed pathways as a continuum of upstream events connected by arrows that may lead to downstream events observed in epidemiologic studies. Evidence supporting these proposed pathways was derived from Sections 7.3 and 7.4 of this ISA, evidence reviewed in the 2013 Pb ISA (U.S. EPA 2013), and recent evidence collected from studies that may not meet the current PECOS criteria but contain mechanistic information supporting these pathways. Discussion of how exposure to Pb may lead to hematological effects contributes to an understanding of the biological plausibility of epidemiologic results. Note that the structure of the biological plausibility section and the role of biological plausibility in contributing to the weight-of-evidence analysis used in the 2013 Pb ISA are discussed below.



ALAD = δ-aminolevulinate dehydratase; Ca^{2+} = calcium ion; Mg^{2+} = Magnesium ion; RBC = red blood cell.

Note: The boxes represent the effects for which there is experimental or epidemiologic evidence related to Pb exposure, and the arrows indicate a proposed relationship between those effects. Solid arrows denote evidence of essentiality as provided, for example, by an inhibitor of the pathway used in an experimental study involving Pb exposure. Dotted arrows denote a possible relationship between effects. Shading around multiple boxes is used to denote a grouping of these effects. Arrows may connect individual boxes, groupings of boxes, and individual boxes within groupings of boxes. Progression of effects is generally depicted from left to right and color coded (white, exposure; green, initial effect; blue, intermediate effect; orange, effect at the population level or a key clinical effect). Here, population-level effects generally reflect results of epidemiologic studies. When there are gaps in the evidence, there are complementary gaps in the figure and the accompanying text below. The structure of the biological plausibility sections and the role of biological plausibility in contributing to the weight-of-evidence analysis used in the 2022 Pb ISA are discussed in Section 7.6.

Figure 7-1 Potential biological plausibility pathways for hematological effects associated with exposure to Pb

Careful review of the available evidence indicates that exposure to Pb has the potential to modulate the hematological parameters leading to decreased RBC survival and function and altered heme synthesis. These deficits converge, promoting the development of anemia, a condition that occurs when the number of RBCs and/or the concentration of Hb in RBCs is abnormally low. Below, evidence from peer-reviewed toxicology studies providing biological plausibility for Pb-associated effects on hematological parameters is reviewed.

7.5.1. Decreased Red Blood Cell Survival and Function

As described below, there is strong evidence that Pb impacts a series of hematological parameters along a cascade of events that results in decreased RBC function and survival, possibly leading to anemia (Figure 7-1). As reviewed in the 2013 Pb ISA, Pb uptake into human RBCs occurs through a passive anion transport mechanism, and once Pb is in the cell, little leaves (Bergdahl et al. 1997; Simons 1993; Simons 1986). While the precise mechanisms responsible for decreasing RBC lifespan and mobility are unknown, occupational Pb exposure has been shown to decrease intracellular free Ca^{+2} levels and decrease Ca^{2+}/Mg^{2+} ATPase activity in RBCs in workers (Abam et al. 2008; Quintanar-Escorza et al. 2007). These changes are associated with fragility and morphological alterations in RBCs in Pb-exposed workers. Pb-induced increases in intracellular Ca^{2+} levels also play a role in the activity of phospholipid scramblases and flippases in RBCs, increasing access to PS by tissue macrophages and triggering splenic sequestration and destruction of RBCs, leading to reduced numbers of RBCs in circulation (Jang et al. 2011). Importantly, Ahyayauch et al. (2018) showed that inhibiting Ca^{2+} increase stimulated by Pb results in decreased flippase activity and prevented destruction of RBCs. This pathway is depicted in Figure 7-1 by solid lines linking increased intracellular Ca^{2+} to increased PS exposure and decreased RBC survival. In addition, phagocytosis of Pb-exposed RBC by human renal proximal tubular cells was mediated by PS (Kwon and Chung 2016). Heme-regulated eIF2α kinase was shown to protect RBC from Pb-induced hemolytic stress in mice (Wang et al. 2015). Pb-induced hemolysis was also documented in other recent studies (Hossain et al. 2015; Mrugesh et al. 2011). Furthermore, Pb exposure reduced the number of RBCs and Hb levels in rats (Ibrahim et al. 2012). Consistent with the pattern seen in epidemiology studies, the effects of Pb exposure on RBC number and Hb level were more pronounced in 3-month-old Wistar rats than in adult animals (Daku et al. 2019). In addition, Cai et al. (2018) reported that Pb administration reduced RBC lifespan in mice. These findings support the conclusion that Pb alters RBC survival and function, consistent with the larger body of evidence showing measures of decreased hematological parameters (i.e., RBC number, Hb, Hct, MCV, and/or MCH) in children (Guo et al. 2021; Kuang et al. 2020; Li et al. 2018; Liu et al. 2015; Liu et al. 2012) and animals (Odo et al. 2020; Andjelkovic et al. 2019; Cai et al. 2018; Corsetti et al. 2017; Lakshmi et al. 2013; Berrahal et al. 2011; Sharma et al. 2010; Wang et al. 2010b; Baranowska-Bosiacka et al. 2009; Massó-González and Antonio-García 2009; Simsek et al. 2009; Massó et al. 2007).

Pb exposure also has the potential to disrupt normal hematopoiesis. Erythropoietin (EPO) is a glycoprotein hormone excreted by the kidney to promote the development of RBCs in bone marrow. As reviewed in the 2006 Pb AQCD, Pb exposure has been observed to alter EPO production in children (Graziano et al. 2004; Factor-Litvak et al. 1999; Factor-Litvak et al. 1998). Available data support the postulation that observed increases in EPO in younger children reflect bone marrow hyperactivity to counteract RBC destruction, whereas the lack of EPO elevation in older children may reflect a transitional period in which increasing renal and bone marrow toxicity leads to observed decreases in EPO later in life (U.S. EPA 2006a). In addition to altering levels of a key hormone involved in hematopoiesis, Pb exposure has the potential to alter hematopoiesis by causing cytotoxicity (Alghazal et al. 2008; Marques et al. 2006; Çelik et al. 2005) and senescence (Cai et al. 2018; Nagano et al. 2015) of RBC precursors. Baktybaeva (2011) reported that intraperitoneal injection of Pb acetate resulted in reduced bone marrow hematopoiesis in mice. Furthermore, Pb is known to reduce erythropoiesis, causing anemia in children (Dai et al. 2017; Ahamed et al. 2011). Altered EPO levels and RBC precursor cytotoxicity have the potential to alter the number of RBCs in circulation which may lead toto anemia.

7.5.2. Altered Heme Synthesis

Although the mechanisms that could lead to Pb-induced anemia are not fully understood, as reviewed in EPA's 2006 AQCD (U.S. EPA 2006b), Pb is known to act directly on two enzymes involved in heme synthesis: ALAD and ferrochelatase. ALAD, a cytoplasmic enzyme requiring zinc (Zn) for enzymatic activity, catalyzes the rate-limiting step in heme biosynthesis. Inhibition of ALAD activity has been reported in adults (Wang et al. 2010a) and children (Dai et al. 2017; Wang et al. 2010a; Ahamed et al. 2007) as well as in animal toxicology studies reporting BLLs relevant to this ISA (i.e., 24.7 µg/dL) (Rendón-Ramirez et al. 2007; Terayama et al. 1986). ALAD activity was also reduced in animal studies reporting BLLs higher than those meeting the PECOS criteria in this ISA (Mani et al. 2020; Velaga et al. 2014; Whittaker et al. 2011; Gautam and Flora 2010; Lee et al. 2005). Pb exposure has also been shown to inhibit ferrochelatase, a mitochondrial iron (Fe)-sulfur (S)containing enzyme that incorporates Fe^{2+} into protoporphyrin IX to create heme (Rendón-Ramirez et al. 2007). Pb inhibits the insertion of Fe^{2+} into the protoporphyrin ring and instead, Zn is inserted into the ring creating Zn-protoporphyrin (ZPP). Evidence for altered heme synthesis is supported by a large body of evidence collected from occupationally exposed adults (Ukaejiofo et al. 2009; Khan et al. 2008; Patil et al. 2006; Karita et al. 2005), children (Queirolo et al. 2010; Shah et al. 2010; Olivero-Verbel et al. 2007; Riddell et al. 2007), and Pb-exposed experimental animal models (Andjelkovic et al. 2019; Cai et al. 2018; Berrahal et al. 2011; Baranowska-Bosiacka et al. 2009; Massó-González and Antonio-García 2009; Simsek et al. 2009; Rendón-Ramirez et al. 2007; Marques et al. 2006; Terayama et al. 1986) reporting decreased Hb concentrations in association with Pb exposure or increased BLLs. Further demonstrating the role Pb plays in the activity of these important enzymes, chelation therapy restored ALAD activity and reduced ZPP levels in blood harvested from rats exposed to Pb via drinking water for 90 days (Ata et al. 2018).

Oxidative stress is caused by an imbalance between production and elimination of reactive oxygen species (ROS) in cells or tissues that exceed the capacity of antioxidant defense mechanisms. ROS are unstable, highly reactive molecules formed from molecular oxygen and include, for example, superoxide (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2). Although ROS play an important role in healthy biological systems (e.g., cell signaling, cellular differentiation, immune responses), unregulated ROS can cause direct and indirect damage to nucleic acids, proteins, and lipids leading to cytotoxicity, tissue injury, and even disruption of normal physiology (Auten and Davis 2009). Oxidative stress is involved in both arms of the pathway leading to anemia shown in Figure 7-1, including effects on RBC MDA, SOD, CAT, GSH, and GPx levels (Kharoubi et al. 2008; Lee et al. 2005). Effects of Pb exposure on measures of oxidative stress—including MDA and decreased SOD, CAT, GR, and GSSG—were also observed in vitro (Ciubar et al. 2007; Coban et al. 2007).

Supporting the role of oxidative stress in the development of anemia, administration of antioxidants reduced the effects of Pb exposure on levels of GSH (Alcaraz-Contreras et al. 2011), MDA (Alcaraz-Contreras et al. 2011) and Hb (Farooq et al. 2016; Sajitha et al. 2016; Sarkar et al. 2015; Eshginia and Marjani 2013) and reduced the effects of Pb exposure on SOD activity (Eshginia and Marjani 2013), ROS production (Nagano et al. 2015; Sarkar et al. 2015), hematopoietic stem cell (HSC) number (Nagano et al. 2015), HSC colony formation (Cai et al. 2018), HSC senescence markers (Cai et al. 2018; Nagano et al. 2015), RBC number (Farooq et al. 2016; Sajitha et al. 2016; Sarkar et al. 2015), and PS exposure (Sarkar et al. 2015) in animal studies. Conflicting evidence on the effects of antioxidant treatment on ALAD activity was reported in the literature (Sajitha et al. 2016; Alcaraz-Contreras et al. 2011), whereas the evidence for effects of Pb exposure on Hb levels was consistent; thus, a solid arrow connects oxidative stress to decreased RBC Hb content in Figure 7-1. Further demonstrating the role of oxidative stress in the development of anemia, decreased ALAD activity results in the accumulation of δ-aminolevulinic acid (δ-ALA) in blood and urine, where it undergoes tautomerization and autoxidation. Oxidized δ-ALA leads to the generation of ROS (i.e., O_2^-, OH, H_2O_2, and an aminolevulinic acid [ALA]) radicals (Hermes-Lima et al. 1991; Monteiro et al. 1991; Monteiro et al. 1989; Monteiro et al. 1986). Reflecting the strength of the evidence described above, the pathway connecting oxidative stress to altered hematopoiesis, cytotoxicity/senescence RBC precursor cells, and decreased RBC survival is depicted as a solid line.

Decreased RBC Hb content and oxidative stress associated with Pb exposure have been demonstrated to alter RBC function. This conclusion is supported by direct evidence for binding of Pb to key enzymes in the heme synthesis pathway, Pb-induced oxidative stress resulting in decreased RBC Hb content and effects on hematopoiesis, RBC precursor cells, and RBC survival. Decreased RBC number coupled with impaired RBC function, if of sufficient magnitude, leads to Pb-induced anemia.

7.6 Summary and Causality Determination

7.6.1. Causality Determination for Red Blood Cell Survival and Function

The 2013 Pb ISA presented causality determinations for two groups of hematological endpoints: heme synthesis and RBC survival and function. Although there are enzymes and hematological parameters that are distinct indicators of these processes, the potential biological plausibility pathways in which exposure to Pb may result in hematological effects demonstrate a spectrum of events, which can be challenging to attribute to a unique line of evidence (Figure 7-1). For example, altered heme synthesis can decrease Hb levels, which in turn has been shown to alter RBC function. Because of this interconnectedness, this assessment presents a single causality determination for Pb exposure and heme synthesis and RBC survival and function. This approach allows for a more holistic evaluation of inter-related health endpoints, including a discussion of how all individual lines of evidence contribute to the overall causality determination. The key evidence, as it relates to the causal framework, is outlined below, and summarized in Table 7-1.

7.6.2. Evidence for Red Blood Cell Survival and Function

The 2013 Pb ISA concluded that there is a "causal relationship" between Pb exposure and decreased RBC survival and function (U.S. EPA 2013). This causality determination was made on the basis of a strong body of evidence from experimental animal studies demonstrating that Pb exposures alter several hematological parameters (e.g., Hb, Hct, MCV, MCH), induce oxidative stress (e.g., alter antioxidant enzyme activities [SOD, CAT, GPx], decrease cellular GSH, and increase lipid peroxidation), and increase cytotoxicity in RBC precursor cells in rodents exposed to various forms of Pb via drinking water and gavage resulting in BLLs ≤ 30 µg/dL (Molina et al. 2011; Baranowska-Bosiacka et al. 2009; Lee et al. 2005). Consistent results were observed in several additional studies in rodents that did not report BLLs. Epidemiologic evidence was coherent with results from the evaluated toxicological studies but was subject to more uncertainties. Notably, the epidemiologic evidence consisted of cross-sectional studies that were conducted in populations with higher mean Pb exposures (i.e., BLLs >10 µg/dL), did not thoroughly consider potential confounders, and lacked rigorous statistical methodology. These limitations precluded strong conclusions on the directionality of effects; the level, timing, frequency, and duration of Pb exposure that contributed to the observed associations; and whether the observed associations are independent of potential confounders. Although there were substantial uncertainties in the epidemiologic evidence, animal toxicological evidence established a clear basis for temporality of exposure to Pb and effects on RBCs. Additional support for these findings was provided by toxicological and epidemiologic studies demonstrating increased intracellular calcium concentrations, decreased Ca^{2+}/Mg^{2+} ATPase activity, and increased PS exposure, establishing biologically plausibility for Pb-induced changes in RBC survival.

Although limited in number, recent PECOS-relevant animal toxicological studies continue to support the findings from the last review. The most consistent evidence comes from studies that report decreased Hb levels in rodents following Pb exposures, resulting in BLLs ranging from 7.5 to 14.7 µg/dL (Andjelkovic et al. 2019; Cai et al. 2018; Berrahal et al. 2011). Other recent toxicological studies noted Pb-induced decrements in Hct (Andjelkovic et al. 2019), packed cell volume (PCV) (Berrahal et al. 2011), and hematopoiesis (Andjelkovic et al. 2019). Recent epidemiologic studies expand on the evidence presented in the 2013 Pb ISA and are coherent with the experimental evidence. Although the recent studies are also cross-sectional, they include populations with much lower BLL means (<10 µg/dL) and include more robust adjustment for potential confounding, addressing important uncertainties from the last review. The most consistent epidemiologic evidence indicates an association between increased BLL and decreased Hb levels in children (Section 7.3.1), which is in line with the evidence from recent experimental animal studies. While the clinical relevance of small mean decrements in Hb across exposure quintiles is unclear, a few of the recent epidemiologic studies observed increases in the odds of prevalent anemia in children associated with increasing quantiles of BLLs (Guo et al. 2021; Li et al. 2018). Recent epidemiologic studies of Hb in adults were more limited in number and less consistent than those in children. Additionally, the relatively few studies examining RBC counts were also inconsistent.

7.6.3. Evidence for Heme Synthesis

The 2013 Pb ISA concluded there is a "causal relationship" between Pb exposure and altered heme synthesis (U.S. EPA 2013). This determination was based on a small but consistent body of studies in adult animals reporting that Pb exposures via drinking water and gavage (resulting in BLLs relevant to this ISA) for 15 days to 9 months decreased ALAD (Rendón-Ramirez et al. 2007; Terayama et al. 1986) and ferrochelatase activities (Rendón-Ramirez et al. 2007). Notably, Rendón-Ramirez et al. (2007) observed effects on ALAD and ferrochelatase activities in albino Wistar rats at mean BLLs of 24.7 µg/dL after Pb administration drinking water for 15 or 30 days. Supporting this toxicological evidence was a larger body of ecotoxicological studies that demonstrate altered heme synthesis in Pb-exposed aquatic and terrestrial invertebrates and vertebrates. Ecological evidence from previous reviews consistently observed Pb-induced ALAD inhibition in multiple species, including birds and fish (U.S. EPA 2013, 2006b). Cross-sectional epidemiologic studies provided supporting evidence that concurrent elevated BLLs are associated with decreased ALAD and ferrochelatase activities in both adults and children. However, the majority of these studies are limited by the lack of rigorous methodology and consideration of potential confounding. Although there were limitations in the epidemiologic evidence, some studies in children did control for or consider potential confounding, and effects in adults and children in these studies are coherent with effects observed in animal toxicological studies.

The relationship between Pb exposure and altered heme synthesis was further supported by cross-sectional epidemiologic studies indicating that increased BLLs were associated with decreased Hb in children and occupationally exposed adults. These findings were consistent with several toxicological

studies that observed decreased Hb levels in laboratory animals exposed to Pb. Decreased Hb levels can be a direct indicator of decreased heme synthesis.

Recent PECOS-relevant studies are limited in number and focus mainly on Hb levels but continue to provide support for Pb-related alterations in heme synthesis. Notably, recent epidemiologic studies indicating an association between increased BLLs and decreased Hb include more robust statistical methods, expanded consideration of potential confounders, and populations with much lower BLLs than the studies included in the previous reviews (mean or median BLLs ranging from 3.04 to 8.38 µg/dL; Section 7.3.1). The recent epidemiologic evidence is coherent with recent toxicological studies, which observed Hb decrements in Pb-exposed mice (Andjelkovic et al. 2019; Cai et al. 2018). While the cross-sectional nature of the epidemiologic studies introduces uncertainty about the temporality of the exposure and outcome, animal toxicological evidence establishes clear temporality of exposure to Pb and altered heme synthesis.

7.6.4. Causality Determination

In summary, there is coherent evidence across toxicological and epidemiologic studies that Pb exposures alter several hematological parameters, decrease enzyme activity related to heme synthesis, and increase RBC oxidative stress. Although all evaluated epidemiologic studies are cross-sectional, toxicological studies establish temporality between exposure to Pb and effects on heme synthesis and RBCs. Additionally, recent epidemiologic and animal studies continue to demonstrate evidence of a causal relationship at relevant BLLs, and recent epidemiologic studies address uncertainties from previous reviews by expanding adjustment for potential confounders and using more robust statistical methods (i.e., multivariable regression models). Because of the contribution of bone Pb levels to concurrent BLLs, associations with concurrent BLLs may reflect an effect of past and/or recent Pb exposures. Therefore, there is uncertainty regarding the timing, duration, and level of Pb exposure associated with observed hematological effects in children and adults. Biological plausibility for the observed associations is provided by toxicological and epidemiologic studies demonstrating increased intracellular calcium concentrations, decreased Ca^{2+}/Mg^{2+} ATPase activity, and increased PS exposure, which collectively can lead to fragility, morphological alterations in RBCs, and RBC destruction. **Taken together, there is sufficient evidence to conclude that there is a causal relationship between Pb exposure and hematological effects, including altered heme synthesis and decreased RBC survival and function.**

Table 7-1 Summary of evidence indicating a causal relationship between Pb exposure and hematological effects

Rationale for Causality Determination[a]	Key Evidence[b]	Key References[b]	Pb Biomarker Levels Associated with Effects[c]
Red Blood Cell Survival and Function			
Consistent evidence from toxicological studies with relevant exposures	Large body of studies with generally consistent findings for decreased RBC survival and function in rodents:	See Section 7.3.2	
			Mean BLLs (± SD):
	Decreased plasma Hb concentration	Cai et al. (2018)	9.3 ± 0.98 μg/dL
		Andjelkovic et al. (2019)	29.0 ± 43.1 μg/dL
		Berrahal et al. (2011)	12.67 ± 1.68 μg/dL PND 40; 7.49 ± 0.78 ug/dL PND 65
		Sharma et al. (2010)	1.72 ± 0.02 μg/dL
		Massó-González and Antonio-García (2009)	22.8 ± 0.50 μg/dL
		Baranowska-Bosiacka et al. (2009)	7.11 ± 1.7 μg/dL
	Decreased Hct	Andjelkovic et al. (2019)	29.0 ± 14.7 ug/dL
		Massó et al. (2007)	22.8 ± 0.50 μg/dL
		Massó-González and Antonio-García (2009)	22.8 ± 0.50 μg/dL
		Berrahal et al. (2011)	12.67 ± 1.68 μg/dL PND 40; 7.49 ± 0.78 μg/dL PND 65
	Decreased RBCs	Andjelkovic et al. (2019)	29.0 ± 14.7 μg/dL

Rationale for Causality Determination[a]	Key Evidence[b]	Key References[b]	Pb Biomarker Levels Associated with Effects[c]
		Cai et al. (2018)	9.3 ± 0.98 μg/dL
		Sharma et al. (2010)	1.72 ± 0.02 μg/dL
	Decreased PCV	U.S. EPA (2013)	
	Increased eryptosis	U.S. EPA (2013)	
		U.S. EPA (2006b)	
	Decreased hematopoiesis	U.S. EPA (2013)	
		U.S. EPA (2006b)	
	Increased oxidative stress	In vitro:	
		Ciubar et al. (2007)	
		Coban et al. (2007)	
		Shin et al. (2007)	
Consistent evidence from multiple epidemiologic studies of children with relevant BLLs provides coherence with toxicological evidence.	Cross-sectional studies provide support for experimental evidence with consistent associations between blood Pb and decreased in Hb in children. Recent studies adjusted for a number of relevant potential confounders, including age, sex, BMI, SES factors, and nutrition.		**Mean BLLs:**
		Guo et al. (2021)	3.07–3.21 μg/dL
		Kuang et al. (2020)	3.04 μg/dL
		Li et al. (2018)	8.38 μg/dL (Median)
		Liu et al. (2015)	7.33 μg/dL
		Liu et al. (2012)	4.30 μg/dL (Median)

Rationale for Causality Determination[a]	Key Evidence[b]	Key References[b]	Pb Biomarker Levels Associated with Effects[c]
	Cross-sectional studies provide generally consistent evidence for associations between blood Pb and altered hematological parameters (e.g., RBC counts, Hct, MCV, and MCH), measures of oxidative stress (e.g., SOD, CAT, GPx, GSH, and lipid peroxidation), and hematopoiesis (e.g., decreased erythropoietin). Evidence base limited by lack of adjustment for potential confounders and populations with higher BLLs.	(U.S. EPA 2013)	>10 µg/dL
Biological Plausibility Altered RBC membrane ion transport PS exposure	Evidence of increased $[Ca^{2+}]_i$ and decreased Ca^{2+}/Mg^{2+}ATPase activity in the RBCs of exposed workers. $[Ca^{2+}]_i$ levels highly correlated with blood Pb even among unexposed controls. $[Ca^{2+}]_i$ levels increased in RBCs from healthy volunteers when exposed in vitro to Pb. $[Ca^{2+}]_i$ associated with increased RBC fragility and alterations in RBC morphology. Consistent evidence from in vivo and in vitro studies that Pb exposure increases PS exposure on RBC membranes via modulation of $[Ca^{2+}]_i$ concentrations. Increased PS exposure leads to eryptosis and phagocytosis by macrophages.	See Section 7.5.2	
Heme Synthesis			
Consistent evidence in animals with relevant exposures	A small, but consistent toxicology evidence base indicates decreased heme synthesis in rodents with relevant Pb concentrations and routes of exposure.	Rendón-Ramirez et al. (2007) Terayama et al. (1986)	**BLL:** 24.7 ± 2.4 µg/dL Exposures: 500–5,000 ppm in drinking water, 15–30 days as adults
		Ahamed et al. (2006)	**Mean BLL:** 7.40 and 13.27 µg/dL

Rationale for Causality Determination[a]	Key Evidence[b]	Key References[b]	Pb Biomarker Levels Associated with Effects[c]
Coherence in a limited number of epidemiologic studies with relevant BLLs	Cross-sectional studies that considered potential confounding by age, sex, urban/rural residence, height, weight, BMI found consistent associations with lower ALAD and ferrochelatase activities in children.	Ahamed et al. (2007)	**BLL:** >10 µg/dL compared with <10 µg/dL
	Concurrent BLL associated with lower ALAD and higher ZPP in adults with consideration for potential confounding by age, sex, smoking status, and alcohol use.	Wang et al. (2010a)	**Mean BLL**: 6.71 µg/dL
Support from toxicological and epidemiologic evidence for decreases in Hb, a direct marker of decreased heme synthesis	Consistent evidence in animals with relevant Pb exposures for decreases in Hb levels.	Baranowska-Bosiacka et al. (2009). Sharma et al. (2010) Section 7.4.2	**Adult animals:** BLL 1.7 µg/dL after 40-day Pb exposure
	Consistent associations between concurrent BLLs and decreased Hb in children. Associations observed at low BLLs with thorough consideration of potential confounders.		**Mean BLLs:**
		Guo et al. (2021)	3.07–3.21 µg/dL
		Kuang et al. (2020)	3.04 µg/dL
		Li et al. (2018)	8.38 µg/dL (Median)
		Liu et al. (2015)	7.33 µg/dL
		Liu et al. (2012)	4.30 µg/dL (Median)
Biological Plausibility	Altered Ion Status: Evidence that Pb competitively inhibits the binding of Zn ions necessary for ALAD activity. Pb also inhibits the incorporation of Fe^{2+} into protoporphyrin IX by ferrochelatase, resulting in Zn-protoporphyrin production.	See Section 7.5.2	

Rationale for Causality Determination[a]	Key Evidence[b]	Key References[b]	Pb Biomarker Levels Associated with Effects[c]

ALAD = δ-aminolevulinate dehydratase; BLL = blood lead level; BMI = body mass index; Ca^{2+} = calcium; CAT = catalase; Fe^{2+} = iron; GPx = glutathione peroxidase; GSH = glutathione; Hb = hemoglobin; Hct = hematocrit; i = inorganic; MCV = mean corpuscular volume; Mg^{2+} = magnesium; Pb = lead; PCV = packed cell volume; PND = postnatal day; PS = phosphatidylserine; RBC = red blood cell; SES = socioeconomic status; SOD = superoxide dismutase; Zn = zinc; ZPP = Zn-protoporphyrin.

[a]Based on aspects considered in judgments of causality and weight of evidence in causal framework in Table I and Table II of the Preamble to the ISAs (U.S. EPA 2015).

[b]Describes the key evidence and references, supporting or contradicting, contributing most heavily to causality determination and, where applicable, to uncertainties or inconsistencies. References to earlier sections indicate where the full body of evidence is described.

[c]Describes the Pb biomarker levels at which the evidence is substantiated.

1

7.7 Evidence Inventories—Data Tables to Summarize Study Details

Table 7-2 Epidemiologic studies of exposure to Pb and hematological effects

Reference and Study Design	Study Population	Exposure Assessment	Outcome	Confounders	Effect Estimates and 95% CIs[a]
Children					
†Guo et al. (2021) Guangdong China 2014-2017 Cross-sectional	Guangdong Women and Children's Hospital n: 17486 Children 0–5 yr old visiting hospital for routine health examination	Blood BLLs were measured using atomic absorption spectrometry Age at Measurement: 0–5 yr Means: Males: 3.21 µg/dL; Females: 3.07 µg/dL	Hb Hb (g/L) measured using an automated hematology analyzer Age at Outcome: 0–5 yr	Age and sex	**Hb** **Mean Difference (g/L)** *Q1 (<1.61)* Reference *Q2 (1.61–2.44)* −0.05 (−0.51, 0.40) *Q3 (2.44–3.33)* −0.02 (−0.48, 0.43 *Q4 (3.33–4.50)* −0.48 (−0.94, −0.04) *Q5 (>4.50)* −1.25 (−1.71, −0.78) **Anemia (OR)** *Q1 (<1.61)* Reference *Q2 (1.61–2.44)* 1.08 (0.94, 1.23) *Q3 (2.44–3.33)* 1.16 (1, 1.33) *Q4 (3.33–4.50)* 1.25 (1.07, 1.43) *Q5 (>4.50)*

Reference and Study Design	Study Population	Exposure Assessment	Outcome	Confounders	Effect Estimates and 95% CIs[a]
					1.45 (1.26, 1.67)
†Kuang et al. (2020) Nanjing China 2012 Cross-sectional	n: 395 Convenience sample of children 7–11 yr old	Blood Blood Pb was measured in venous whole blood using ICP-MS Age at Measurement: 7–11 yr old Mean: 3.04 µg/dL; Median: 2.61 µg/dL	Hematological Parameters RBC, Hb, Hct, MCV, MCH, and MCHC measured by a whole cell analyzer Age at Outcome: 7–11 yr old	Picky eaters and passive smoking (age, gender, parents' education, and parents' occupation also considered)	**RBC Count (10^{12}/L)** *Boys:* 0.02 (-0.01, 0.04) *Girls:* 0.01 (-0.02, 0.03) **Hb (g/L)** *Boys:* -0.12 (-0.22, -0.02) *Girls:* -0.08 (-0.23, 0.07) **Hct (%)** *Boys:* -0.04 (−0.08, −0.01) *Girls:* −0.02 (−0.07, 0.02) **MCV (fL)** −0.04 (−0.06, −0.02) **MCH (pg)** −0.01 (−0.02, −0.00) **MCHC (g/L)** 0.26 (−0.64, 1.15)
†Liu et al. (2012) Changzhou City China Cross-sectional	China Jintan Child Cohort Study n: 140 Convenience sample of preschool age children	Blood Blood Pb was measured in whole blood using GFAAS Age at Measurement: Median age: 3 yr old Median: 4.3 µg/dL Maximum: 11.4 µg/dL	Hb Hb measured in whole blood using a photoelectric colorimeter Age at Outcome: Median age: 3 yr old	Age, sex, height, weight, iron deficiency	**Hb (g/dL)*** *Full Population* −0.096 (−0.18, −0.012) *Blood Pb < 10 µg/dL* −0.174 (−0.27, −0.078)

Reference and Study Design	Study Population	Exposure Assessment	Outcome	Confounders	Effect Estimates and 95% CIs[a]
†Li et al. (2018) Hubei and Hunan Provinces China 2012-2017 Cross-sectional	Blood Lead Intervention Program n: 758 Children Ages 5–8 yr recruited from four counties in two provinces One county in each province had high environmental Pb levels (battery plant and mining)	Blood Blood Pb was measured in venous whole blood using GFAAS Age at Measurement: 5–8 yr Median: 8.38 µg/dL 75th: 13.51 µg/dL 90th: 18.77 µg/dL 95th: 21.82 µg/dL	Hematological Parameters Hb, MCH, RBCs, and Plt measured in venous whole blood using an automated hematology analyzer Age at Outcome: 5–8 yr	Age, sex, BMI, environmental Pb exposure level, and serum iron, zinc, and calcium	**ORs** **Decreased Hb (<115 g/L)** 1.05 (1.00, 1.11) **Decreased RBC (<4 × 10^{12}/L for boys; <3.5 × 10^{12}/L for girls)** 1.11 (1.05, 1.16) **Decreased Plt (<100 × 10^{9}/L)** 1.11 (1.05, 1.16) **Decreased MCH (<27 pg)** 1.11 (1.05, 1.16)
†Liu et al. (2015) Guiyu, Chendian, and Chaonan China 2006–2011 Cross-sectional	n: 855 Children 3–7 yr old from e-waste processing area or control industrial areas without high environmental Pb exposures	Blood Blood Pb was measured using GFAAS. Blood Pb was divided by Hct as a fraction of the whole blood to estimate erythrocyte Pb Age at Measurement: 3–7 yr old Median: Blood Pb: 7.33 µg/dL; Erythrocyte Pb: 19.3 µg/dL	Hb Hb, MCH, RBCs, and Plt measured in venous whole blood using an automated hematology analyzer Age at Outcome: 3–7 yr old	Age, sex, residence area, and SES	**Hb (g/L)** **Mean Difference** *Q1 (5.98–13.52)** Reference *Q2 (13.52–19.35)** −0.02 (-1.89, 1.52) *Q3 (19.35–28.42)** -3.01 (−4.71, 1.31) *Q4 (28.42–101.01)** −3.97 (−5.68, −2.27) *Erythrocyte Pb (µg/dL)
Adults					

Reference and Study Design	Study Population	Exposure Assessment	Outcome	Confounders	Effect Estimates and 95% CIs[a]
†Park and Lee (2013) South Korea 2008-2010 Cross-sectional	KNHANES n: 4522 General population, ≥20 yr old	Blood Blood Pb was measured in venous whole blood using GFAAS Age at Measurement: ≥20 yr Geometric Means: Males: 2.46 µg/dL; Females: 1.98 µg/dL	Hb Blood Hb (g/dL) measured using an automated hematology analyzer Age at Outcome: ≥20 yr	Age, BMI, education, smoking and drinking status, and rural/urban residence	**Hb (g/dL)*** *Men* 0.04 (0.03, 0.06) *Women* 0.04 (0.02, 0.06) *Not standardized. Per ln(Pb) increase
†Kim and Lee (2013) South Korea 2008-2010 Cross-sectional	KNHANES n: 5951 General population, ≥20 yr old	Blood Blood Pb was measured in whole blood using GFAAS. Blood Pb was divided by Hct as a fraction of the whole blood to estimate erythrocyte Pb Age at Measurement: ≥20 yr old Median: Blood Pb: 2.31 µg/dL; Erythrocyte Pb: 5.4 µg/dL 75th: Blood Pb: 3.01 µg/dL; Erythrocyte Pb: 6.9 µg/dL	Hb Hb measured in whole blood using an automated hematology analyzer Age at Outcome: ≥20 yr old	Sex, age, obesity, residence area, education level, smoking and drinking status, serum ferritin, and serum creatinine	**Hb (g/L)** **Mean Difference** *Q1 (<1.73)* Reference *Q2 (1.73–2.31)* 0.13 (0.03, 0.23) *Q3 (2.31–3.01)* 0.33 (0.23, 0.42) *Q4 (>3.01)* 0.42 (0.30, 0.53) *Q1 (<4.1)** Reference *Q2 (4.1–5.4)* −0.06 (0.15, 0.03) *Q3 (5.4–6.9)* −0.06 (-0.15, 0.03) *Q4 (>6.9)* −0.14 (−0.25, −0.04) *Erythrocyte Pb (µg/dL)

Reference and Study Design	Study Population	Exposure Assessment	Outcome	Confounders	Effect Estimates and 95% CIs[a]
†La-Llave-León et al. (2015) Durango Mexico 2007-2008 Cross-Sectional	n: 292 Pregnant women, 14–41 yr old	Blood Blood Pb was measured in venous whole blood using GFAAS Age at Measurement: 14–41 yr old Mean: 2.79 µg/dL	Hematological Parameter RBC, Hb, Hct, MCV, MCH, and MCHC measured using an automated hematology analyzer Age at Outcome: 14–41 yr old	BMI, gestational age, age, parity, gestations, and household monthly income per person	**RBC Count (× 10^6 µg/dL)** 0.034 (0.013, 0.056)

BMI = body mass index; BW = body weight; CI = confidence interval; e-waste = electronic waste; GFAAS = graphite furnace atomic absorption spectroscopy; Hb = hemoglobin; Hct = hematocrit; ICP-MS = inductively coupled plasma mass spectrometry; KNHANES = Korean National Health and Nutrition Examination Survey; ln = natural log; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; OR = odds ratio; Pb = lead; Plt = platelet; PND = postnatal day; Q = quartile; RBC = red blood cell; RDW = red blood cell distribution width; SES = socioeconomic status; yr = years.

[a] Effect estimates are standardized to a 1 µg/dL increase in blood Pb level or a 10 µg/g increase in bone Pb level, unless otherwise noted. For studies that report results corresponding to a change in log-transformed Pb biomarkers, effect estimates are assumed to be linear within the 10th to 90th percentile interval of the biomarker and standardized accordingly.

†Studies published since the 2013 Pb ISA.

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Table 7-3 Animal toxicological studies of Pb exposure and hematological effects

Study	Species (Stock/Strain), n, Sex	Timing of Exposure	Exposure Details (Concentration, Duration)	BLL as Reported (µg/dL)[a]	Endpoints Examined
Berrahal et al. (2011)	**Rat (Wistar)** Control (vehicle), M, n = 12–16 50 mg/L Pb, M, n = 12–16	PND 1 to PND 21: Lactational PND 21 to PND 40 or PND 65: Drinking water	Dams were given 50 mg/L Pb acetate in drinking water until weaning on PND 21. Male offspring received 50 mg/L Pb acetate in drinking water from PND 21 to PND 40 or PND 65. Control animals received tap water.	PND 40: 1.76 ± 0.33 µg/dL for 0 µg/dL 12.67 ± 1.68 µg/dL for 50 mg/L PND 65: 2.06 ± 0.35 µg/dL for 0 µg/dL 7.49 ± 0.78 µg/dL for 50 mg/L	Hct, Hb
Basha et al. (2012)	**Rat (Wistar)** Control (vehicle), M, n = 8 0.2% Pb, M, n = 8	PND 1 to PND 21	Damns given Pb acetate in drinking water or Pb acetate containing water supplemented with 0.02% calcium, zinc, and iron. Control group received deionized water as vehicle (no supplement). Pups exposed through lactation.	PND 45: 0.42 ± 0.04 µg/dL for 0%, 52.5 ± 0.67 µg/dL for 0.2%, 21.1 ± 1.12 µg/dL for 0.2% + supplementation PND 12 mo: 0.56 ± 0.08 µg/dL for 0%, 16.4 ± 1.95 µg/dL for 0.2%, 7.2 ± 0.56 µg/dL for 0.2% + supplementation PND 24 mo: 0.46 ± 0.02 µg/dL for 0%, 12.2 ± 0.76 µg/dL for 0.2%, 4.8 ± 0.5 µg/dL for 0.2% for 0.2% + supplementation	RBC, Hb

Study	Species (Stock/Strain), n, Sex	Timing of Exposure	Exposure Details (Concentration, Duration)	BLL as Reported (µg/dL)[a]	Endpoints Examined
Zou et al. (2015)	**Mouse (ICR)** Control (vehicle), M, n = 10 250 mg/L Pb, M, n = 10	3 wk exposure	Rats received 250 mg/L Pb acetate in redistilled drinking water for 3 weeks. The rats were 30 d old when acquired, but the authors did not specify the age at the time of treatment.	PND 58: 1.8 µg/dL for 0 mg/L 21.7 µg/dL for 250 mg/L	RBC, MCHC
Corsetti et al. (2017)	**Mouse (C57BL.6)** Control (vehicle), M, n = 8 200 ppm Pb, M, n = 8	d 30 to d 75	Mice were exposed via drinking water for 45 consecutive days. Control animals were exposed to drinking water containing acetic acid (1 mL/L).	<5 µg/dL for 0 ppm 21.6 µg/dL for 200 ppm	RBC, Hb, Hct, MCV, MCH, MCHC, RDW %, Plt
Andjelkovic et al. (2019)	**Rat (Wistar)** Control (vehicle), M, n = 8 0.2% Pb, M, n = 6	NR	Rats (250 g), age at time of dosing not reported, were exposed to a single dose of 150 mg Pb/kg BW Pb acetate via oral gavage. Control animals were given "water."	24.9 ±1 9 µg/kg for 0 mg Pb/kg BW (2.6 ± 2.0 µg/dL) 291.2 ± 139 µg/kg for 150 mg Pb/kg BW (29.0 ± 14.7 µg/dL)	RBC, Hb, Hct, MCV, MCH, MCHC, Plt

Study	Species (Stock/Strain), n, Sex	Timing of Exposure	Exposure Details (Concentration, Duration)	BLL as Reported (µg/dL)[a]	Endpoints Examined
Cai et al. (2018)	**Rat (**Sprague Dawley**)** Control (vehicle), M/F, n = 5 0.2% Pb, M/F, n = 5	8–10 wk to 20–22 wk	Rats were 8– 10 wk old when acquired. Whether, or not the rats were allowed to acclimate to the facility prior to study initiation was not reported. The number of males and females not reported. Control animals received tap water. The exposure period was 12 wk, assumed rats were exposed 7 d/wk for a total of 84 d.	20.5 ± 0.68 µg/L for 0% (2.2 ± 6.4 µg/dL) 87.4 ± 9.2 µg/L for 0.2% (9.3 ± 0.98 µg/dL)	Hb, Plt, Erythrocyte life span, RBC

BLL = blood lead level; BW = body weight; d = day; Hb = hemoglobin; Hct = hematocrit; M = male; M/F = male/female; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; mo = month; NR = not reported; Pb = lead; Plt = platelet; PND = postnatal day; RBC = red blood cell; RDW = red blood cell distribution width; wk = weeks.

[a]If applicable, reported values for BLL were converted to µg/dL using WebPlot Digitizer (https://apps.automeris.io/wpd/) and are shown in parenthesis.

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7.8 References

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