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Updated Problem Formulation and Protocol for the Inorganic Arsenic IRIS Assessment

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Supplemental Information – Appendix A

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ABBREVIATIONS

ADME	absorption, distribution, metabolism, excretion
AOPn	Adverse Outcome Pathway network
AR	androgen receptor
As	arsenic
ATO	arsenic trioxide
ATRA	all trans-retinoic acid
ATSDR	Agency for Toxic substances and Disease Registry
BMD	benchmark dose
BMDL	benchmark dose lower confidence limit
CAA	Clean Air Act
CERCLA	Comprehensive Environmental Response, Compensation and Liability Act
CORT	corticosterone
CVD	cardiovascular disease
CWA	Clean Water Act
DAPK	death-associated protein kinase
DCS	diseases of the circulatory system
DMA	dimethylarsinate
DMA(V)	dimethylarsinic acid
DNMTs	altered DNA methyltransferases
DNT	developmental neurotoxicity
E2	estradiol
ECD	electrochemical detection
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
ER	Estrogen receptor
ERα	estrogen receptor alpha
ERK	extracellular signal-regulated kinase
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FSH	follicle-stimulating hormone
GD	Gestational Day
GI	gastrointestinal
GR	glucocorticoid receptor
GRADE	Grading of Recommendations Assessment, Development and Evaluation
GREs	glucocorticoid receptor response elements
GSH	glutathione
HAP	hazardous air pollutants
HCC	hepatocellular carcinoma
HEALS	Health Effects of Arsenic Longitudinal Study
HELF	embryonic lung fibroblasts
HERO	Health and Environmental Research Online
HPA	hypothalamic-pituitary-adrenal
HPLC	high-performance liquid chromatography
HPG	hypothalamic-pituitary-gonadal
IARC	International Agency for Research on Cancer
iAs	inorganic arsenic
ICD	International Classification of Disease
IHD	ischemic heart disease
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
IUR	inhalation unit risk
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	knockout
LECs	lung epithelial cells
LH	luteinizing hormone
LOQ	level of quantitation
MAPKs	mitogen activated protein kinases
MIE	molecular initiating event
miRNA	microRNA
MMA	monomethylarsonate
MOA	mode of action
MR	mineralocorticoids
NA	not applicable
NADPH	nicotinamide adenine dinucleotide phosphate
NCEA	National Center for Environmental Assessment
NF-κB	nuclear factor kappa B
NOEL	no-observed-effect level
NRC	National Research Council
NTP	National Toxicology Program
OHAT	Office of Health Assessment and Translation
OSF	oral slope factor
PBL	peripheral blood lymphocyte
PBMCs	peripheral blood mononuclear cells
PBPK	physiologically based pharmacokinetic
PC	partition coefficient
PECO	populations, exposures, comparators, and outcomes
PND	Postnatal Day
PR	progesterone receptor
PSA	prostate-specific antigen
RAR	retinoic acid receptor
RARE	RAR response element
RfC	inhalation reference concentration
RfD	oral reference dose
ROS	reactive oxygen species
RR	relative risk

RRB	relative risk to background exposure
RRE_{20}	exposure that increases relative risk by 20%
SD	standard deviation
SAM	S-adenosylmethionine
SECs	sinusoidal endothelial cells
SEM	standard error of the mean
SMR	standardized mortality ratio
SOAR	Systematic Omics Analyses Review
SOD	superoxide dismutase
TCEQ	Texas Commission on Environmental Quality
TH	thyroid hormone
TK	toxicokinetics
TR	thyroid hormone receptor
TrxR	thioredoxin reductase
U.S.	United States
VEGF	vascular endothelial growth factor
V_{max}	maximum velocity
WT	wild type
WHO	World Health Organization

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1. INTRODUCTION

The Integrated Risk Information System (IRIS) Program is developing an updated Toxicological Review of Inorganic Arsenic that considers the substantial body of new data and refined methods for hazard assessment and exposure- and dose -response analysis that have emerged since the previous Inorganic Arsenic IRIS assessment was published in 1995. Given the size and complexity of the evidence base for this chemical, input on the scope of this assessment has been sought from the National Research Council (NRC) of the National Academy of Sciences (NAS), Environmental Protection Agency (EPA) program, and regional offices, other federal agencies, and public stakeholders (see Table 1-1) to help focus the scope and objectives of the assessment and ensure it is transparently conducted using the best available scientific data and methods, including systematic review methodology. This current document summarizes the Agency needs for the assessment and presents the refined focus based on problem formulation activities conducted since the last assessment plan released to the NRC in 2015. This document also presents the assessment protocol, which describes methods already used to prioritize health outcomes as part of refining the focus, as well as dose-response and other methods that will be used to complete the assessment. More details on the methods can be found in posters that accompany this protocol (http://hero.epa.gov/index.cfm/project/page/project_id/2211).

Table 1-1. Timeline of EPA activities to update the 1995 inorganic arsenic assessment

History	
1988	EPA published the IRIS Health Hazard Assessment for Inorganic Arsenic.
1991	EPA published a revision to the IRIS RfD.
1995	EPA published a revision to the oral and inhalation cancer assessments.
1999	The National Research Council (NRC), at EPA's request, published the Arsenic in Drinking Water report.
2001	The NRC published Arsenic in Drinking Water 2001 Update.
2003	EPA began updating the 1988 IRIS Toxicological Review.
2005	EPA released the draft IRIS Toxicological Review of Inorganic Arsenic for public comment and external peer review by EPA's Science Advisory Board (SAB).
2007	An expert panel convened by EPA's Science Advisory Board completed a review of key scientific issues included in the draft Toxicological Review and published comments in an advisory report.
2010	EPA released the revised draft IRIS Toxicological Review of Inorganic Arsenic for public comment and external peer review by the SAB.
2010	SAB completed its review of the draft Toxicological Review.
2011	Congress directed EPA to contract with the NRC to review the draft Toxicological Review.
2013 (January)	EPA held a public scoping and problem formulation meeting for refining the draft IRIS Toxicological Review of Inorganic Arsenic.
2013 (March–July)	EPA held eight science issues public webinars.
2013 (May)	EPA submitted a draft Assessment Development Plan and preliminary assessment materials to NRC for review.
2013 (November)	NRC released the interim report, Critical Aspects of EPA's IRIS Assessment of Inorganic Arsenic, and provided recommendations; NRC supported EPA's Assessment Development Plan.
2014 (June)	EPA held a public science meeting to present and solicit public comments on the Assessment Development Plan, preliminary assessment materials, and key science issues.
2015 (December)	EPA briefed the NRC committee on a revised draft Assessment Development Plan with updated dose-response approaches.
2019 (May)	EPA released the protocol for the inorganic arsenic assessment for public comment and NRC review.
2022 (October)	EPA released an updated protocol for the inorganic arsenic assessment.

2. SCOPING AND PROBLEM FORMULATION SUMMARY

2.1. SCOPING SUMMARY

As part of scoping, the Integrated Risk Information System (IRIS) Program works with EPA program offices and regions that have an interest in the assessment to identify their specific needs. A summary of the input received from this outreach effort conducted in 2018 for inorganic arsenic (iAs) is provided in Table 2-1.

Table 2-1. EPA program office or region interest in the inorganic arsenic assessment

EPA program or regional office	Oral	Inhalation	Statutes/regulations and executive orders	Anticipated uses/interest
Office of Land and Emergency Management Regions 1-10	✓	✓	Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Resource Conservation and Recovery Act (RCRA)	iAs has been identified as a contaminant of concern at numerous contaminated waste sites, including more than a hundred National Priority List (NPL) sites. CERCLA authorizes EPA to conduct short- or long-term cleanups at Superfund sites and later recover cleanup costs from potentially responsible parties under section 107. iAs toxicological information may be used to make risk determinations for response actions (e.g., short-term removals, long-term remedial response actions, RCRA Corrective Action).
Office of Water	✓		Safe Drinking Water Act (SDWA) and Clean Water Act (CWA)	The SDWA requires EPA to periodically review the National Primary Drinking Water Regulation (NPDWR) for each contaminant and revise the regulation, if appropriate. iAs toxicological information may be used to inform risk determinations associated with revisiting the NPDWR. Under the CWA, EPA derives 304(a) recommended ambient water quality criteria for the protection of human health. EPA has an existing 304(a) criteria for arsenic, and updated toxicity information could inform any update to criteria.

2.2. BACKGROUND

Inorganic arsenic is a naturally occurring element widely distributed throughout Earth's crust. Inorganic arsenic is found in water, food, soil, and air. In addition to natural sources,

industrial activities such as coal combustion and smelting operations can release inorganic arsenic. This prevalence increases the potential for human exposure; therefore, characterizing the human health impacts of inorganic arsenic exposure is important to Agency stakeholders. As the inorganic arsenic species found most frequently in the environment, As (III) and As(V) were considered in the assessment.

Oral exposure is the primary route of human environmental exposure to inorganic arsenic. The exposure occurs through the dietary intake of arsenic-contaminated food or drinking water, incidental ingestion of soil or sediments containing arsenic. In the cases of fetuses and infants, through transplacental and lactational exposures. Inorganic arsenic is found in foods such as meats, poultry, dairy products, and cereal (IARC, 2009). A portion of the arsenic in foods is found as organic compounds with covalently bound arsenic that originates from inorganic arsenic in water and/or soils.

For the general population, inhalation of inorganic arsenic from the air is not usually a primary route of exposure. However, inhalation can be the primary route of exposure in occupational settings. Higher levels of inhalation exposure to inorganic arsenic have been observed in workers and some residents in areas where there are smelters, mines, and/or arsenical chemical factories. Previous assessments have reported that cigarette smokers can be exposed up to 10 µg of arsenic/day (IARC, 2009); (ATSDR, 2007), although levels of arsenic in cigarettes are reported to have been significantly reduced over the years (Caruso et al., 2014); (Marano et al., 2012).

Unlike the environmental exposures, where the health concerns are mainly from oral exposures, occupational exposures can occur via inhalation and dermal contact. Dermal exposure to inorganic arsenic has been investigated as a route of exposure in occupational settings, but these dermal exposures are most likely concurrent with inhalation and oral exposure, making it difficult to determine the effect of dermal exposure alone.

The potential for exposure from multiple routes and sources exists, particularly for workers and populations near work sites. In some worksite scenarios (e.g., mining), sensitive populations such as reproductive-aged men and women have the potential for increased inorganic arsenic exposure from aggregate exposure at many levels, including exposure from different media within the oral route, across routes of exposure, and in occupational and nonoccupational settings.

The existing IRIS oral reference dose (RfD) for inorganic arsenic is 0.0003 mg/kg-day, based on hyperpigmentation, keratosis, and possible vascular complications observed in many adult residents exposed to arsenic in a Blackfoot disease-endemic area in southwest Taiwan. An inhalation reference concentration (RfC) for inorganic arsenic was not derived (U.S. EPA, 1995). EPA concluded that inorganic arsenic is a human carcinogen via both the oral and inhalation routes of exposure, and cancer risk estimates were calculated. The cancer oral slope factor (OSF) for inorganic arsenic is 1.5 per mg/kg-day based on skin cancers observed in the large southwest Taiwanese cohort referenced above (U.S. EPA, 1995). The cancer inhalation unit risk (IUR) for inorganic arsenic is 0.0043 per µg/m^3 based on respiratory cancer mortality observed in a cohort of

Anaconda, MT smelter workers. This IUR estimates an increase in cancer risk of 1/1,000,000 cases at an arsenic air concentration of 0.0002 μg/m^3 assuming continuous lifetime exposure (U.S. EPA, 1995).

2.3. UPDATED SCOPING AND PROBLEM FORMULATION

In December 2011, EPA received direction from Congress, through the Consolidated Appropriations Act (U.S. Congress, 2011), to contract with the NRC to conduct a review of EPA's draft inorganic arsenic assessment considering both cancer and noncancer hazards from oral exposure. The first phase of the NRC review began in July 2012 and was completed in November 2013. As part of the first phase of the NRC review, EPA provided the NRC draft materials for comment. These draft materials included planning and scoping documents, as well as a draft assessment development plan outlining proposed approaches for literature searches, literature evaluation, hazard identification, and mode-of-action (MOA) and dose-response analyses. In accordance with this Congressional mandate, the NRC provided recommendations to EPA for developing the draft assessment (NRC, 2013). The most recent EPA update to the NRC with a draft Assessment Plan occurred in 2015 (U.S. EPA, 2015). Major past NRC conclusions and recommendations include:

- The committee concluded that human data are expected to be the basis for dose-response analyses but should the epidemiological data in the range of observation be inadequate to meet EPA's needs. MOA data should be used to the extent possible to extrapolate below the observed range (NRC, 2013).

- The committee suggested that health outcomes included in the assessment should be tiered and further prioritized given the volume of data on inorganic arsenic, particularly human data (NRC, 2013). The NRC provided recommendations on three tiers of outcomes, specifically: Tier 1 (evidence of a causal association determined by other agencies and/or in published reviews), Tier 2 (other priority outcomes), and Tier 3 (other endpoints to consider).

- The committee supported EPA's proposal to consider animal and mechanistic data as supporting evidence for determining causality (NRC, 2013).

- The committee agreed with EPA's proposal to conduct dose-response analysis for *causal* or *likely causal* relationships, even in the absence of understanding the potential mode(s)-of-action (NRC, 2013).

- The committee supported EPA's plan to conduct feasibility analyses to determine whether the available MOA evidence is expected to be useful for informing the dose-response of health outcomes classified as having a *causal* or *likely causal* relationship with arsenic (NRC, 2013).

- The committee supported EPA's dose-response meta-analysis approach for epidemiological studies (NRC, 2013).

- The committee agreed with use of the physiologically-based pharmacokinetic (PBPK) model by (El-Masri and Kenyon, 2008) to understand the relationship between drinking water and urinary concentrations of arsenic, as presented to the NRC in 2015 (https://www.epa.gov/iris/inorganic-arsenic-meetings-webinars).

The current document presents adjustments to the 2015 Assessment Plan to clarify the scope of the assessment and describe assessment methods. Both systematic review methods implemented already to prioritize health outcomes as part of refining the focus and dose-response and other methods that will be used to complete the assessment are described here. The refined scope presented here was informed by prior science discussions with the NRC, EPA program and regional offices, and other stakeholders.

2.3.1. Prioritizing Health Outcomes for the Inorganic Arsenic Assessment

Hundreds of epidemiological studies on the toxicity of inorganic arsenic have been published for a broad range of cancer and noncancer outcomes, including large-scale longitudinal cohort studies, case-control studies, and cross-sectional studies. Given this abundance of epidemiological evidence and preference for using human data over animal data when available, human data are expected to be the basis for dose-response analyses (NRC, 2013). With respect to the animal data, most adult laboratory animal models appear to be less susceptible to inorganic arsenic than humans when comparative information is available (Lynch et al., 2017a); (Lynch et al., 2017b); (Vahter, 1994); (Vahter and Norin, 1980). Interspecies metabolism differences likely explain the differences in toxicity between animals and humans, with animals requiring higher doses to reach internal doses comparable to those observed in humans. Another potential confounder in animal studies is the high levels of dietary arsenic found in standard laboratory chow (Kozul et al., 2008). Thus, analysis of the epidemiological evidence base has been the basis for prioritizing health outcomes as described below. Animal and mechanistic evidence has been considered as supplemental evidence in the EPA assessment, an approach supported by the NRC (NRC, 2013) and consistent with assessments by others (ATSDR, 2007); (EFSA, 2009); (TCEQ, 2017). The abundance of epidemiological evidence also focuses the cancer mode-of-action analyses of mechanistic evidence to targeted questions of understanding the shape of the dose-response relationship rather than broader questions of applicability of tumor findings in animals to humans (see §2.3.2).

In its 2013 interim report, the NRC categorized several health outcomes into three tiers of outcomes (see Table 2-2), specifically: Tier 1 (evidence of a causal association determined by other agencies and/or in published reviews), Tier 2 (other priority outcomes), and Tier 3 (other endpoints to consider). NRC advised EPA to further refine these categorizations after conducting a more comprehensive analysis. As part of this further refinement, the EPA conducted hazard

analyses in 2015–2016 that considered the strength of the epidemiological evidence for each health outcome, either by relying on conclusions from other assessments or by conducting new systematic reviews of the literature. EPA characterized the strength of the evidence base for these health outcomes into *robust, moderate*, or *slight* categories (see Table 2-2). The methods used to conduct these systematic reviews are described in Section 3, and the results are summarized below in Table 2-2.

The results of the systematic reviews and hazard analyses will be included in the inorganic arsenic assessment and subject to external peer review (or cited, if published in the peer review literature). Briefly, these categories are characterizations for judgments on the extent of support provided by human studies that the health effect(s) result from chemical exposure. Repeated observations of associations by independent studies examining various aspects of exposure or response (e.g., across different exposure settings, dose levels or patterns, populations, and related endpoints) result in a stronger strength of evidence judgement. These terms are differentiated by the quantity and quality of information available to rule out alternative explanations for the results.

Based on those qualitative hazard analyses of the inorganic arsenic literature, the following health outcomes were identified for potential dose-response analyses consideration in the assessment based on a determination of *robust* or *moderate* evidence (see Table 2-2). These outcomes include cancers of the bladder, lung, kidney, liver, and skin; and noncancer effects of inorganic arsenic on the circulatory system (ischemic heart disease, hypertension, and stroke), reproductive system (including pregnancy and birth outcomes), developmental outcomes (including neurodevelopmental toxicity), endocrine system (including diabetes), immune system, respiratory system, and skin. Health outcomes with "slight" evidence (prostate and pancreatic cancer and renal disease) were not further considered for dose-response. These health outcomes generally aligned with those categorized by the NRC as Tier 1 (evidence of a causal association determined by other agencies and/or in published systematic reviews) or Tier 2 (other priority outcomes), except for prostate cancer, which was considered Tier 2 by NRC but *slight* based on the 2015–2018 analyses conducted by EPA that was more comprehensive and systematic.

As described in Chapter 5, the selection of specific studies and data sets for use in dose-response analyses takes into consideration existing EPA guidance and support documents, especially EPA's *Benchmark Dose Technical Guidance* (U.S. EPA, 2012), EPA's *Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002b), *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), and *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA, 2005b)

Table 2-2. Strength of evidence judgments to help prioritize health outcomes of concern for EPA's inorganic arsenic assessment

Health outcome	NRC tier (NRC, 2013)	EPA strength-of-evidence judgement of human evidence of a causal association
NRC Tiers: Tier 1: Evidence of causality; Tier 2: Other priority outcome; Tier 3: Other endpoints to consider		
Lung cancer	Tier 1	**Robust**. Based on NRC Tier 1 and conclusions of "carcinogenic" for lung cancer from other assessments (NTP, 2016); (ATSDR, 2007); (ATSDR, 2016); (IARC, 2004b); (IARC, 2012); (WHO, 2011b); (WHO, 2011a); (Lynch et al., 2017b); (Lynch et al., 2017a).
Bladder cancer	Tier 1	**Robust**. Based on NRC Tier 1 and conclusions of "carcinogenic" for bladder cancer from other assessments or review articles (NTP, 2016); (ATSDR, 2007); (ATSDR, 2016); (IARC, 2004b); (IARC, 2012); (WHO, 2011b); (WHO, 2011a).
Skin cancer	Tier 1	**Robust**. Based on 1995 EPA conclusion of "known carcinogen" based on skin cancer (U.S. EPA, 1995), NRC Tier 1, and conclusions of "carcinogenic" for skin cancer based on other assessments (NTP, 2016); (ATSDR, 2007); (ATSDR, 2016); (IARC, 2012); (WHO, 2011b); (WHO, 2011a).
Ischemic heart disease	Tier 1	**Robust**. Based on systematic review conducted by EPA on diseases of the circulatory system (ischemic heart disease and hypertension/stroke), which is like associations noted in other assessments (ATSDR, 2007); (ATSDR, 2016); (WHO, 2011b); (WHO, 2011a) and meta-analysis[a] (Moon et al., 2013); (Moon et al., 2017a); (Moon et al., 2017b).
Skin lesions	Tier 1	**Robust**. Based on NRC Tier 1 and conclusions from other assessments (ATSDR, 2007); (ATSDR, 2016); (WHO, 2011b); (WHO, 2011a).
Diabetes	Tier 2	**Robust**. Based on systematic review conducted by EPA, which is like associations noted in (ATSDR, 2016), an expert review conducted as part of an NTP workshop (Maull et al., 2012); (Thayer et al., 2012) and a meta-analysis[a] (Wang et al., 2014).
Pregnancy outcomes (fetal and infant morbidity)	Tier 2	**Moderate**. Based on systematic review conducted by EPA on pregnancy and birth outcomes (fetal growth, prematurity, and infant growth in the first 5 yr of life), which is similar to associations noted in (ATSDR, 2016) and meta-analysis[a] by (Quansah et al., 2015).
Pregnancy outcomes (fetal loss, stillbirth, and neonatal mortality)	Tier 3	**Moderate**. Based on systematic review conducted by EPA on pregnancy and birth outcomes (fetal loss and infant mortality in the first 5 yr of life), which is similar to associations noted in (ATSDR, 2016), review by (Bloom et al., 2010), and a meta-analysis[a] by (Quansah et al., 2015).
Hypertension/stroke[b]	Tier 3	**Robust**. Based on systematic review conducted by EPA on diseases of the circulatory system (including ischemic heart disease and hypertension/stroke), which is similar to associations noted in (ATSDR, 2016), review by (Abhyankar et al., 2012), and meta-analysis[a] (Moon et al., 2013); (Moon et al., 2017a); (Moon et al., 2017b).
Renal cancer	Tier 2	**Moderate**. Based on systematic review conducted by EPA, which is similar to associations noted in (IARC, 2004b); (IARC, 2012) and (ATSDR, 2016).

Health outcome	NRC tier (NRC, 2013)	EPA strength-of-evidence judgement of human evidence of a causal association
Nonmalignant respiratory disease	Tier 2	**Moderate**. Based on systematic review conducted by EPA, which is similar to associations noted in (ATSDR, 2016).
Neurodevelopmental toxicity	Tier 2	**Moderate**. Based on systematic review conducted by EPA, which is similar to associations noted in (ATSDR, 2016).
Immune effects	Tier 2	**Moderate**. Based on systematic review conducted by EPA, which is similar to associations noted in (ATSDR, 2016).
Liver cancer	Tier 3	**Moderate**. Based on systematic review conducted by EPA, which is similar to associations noted in (IARC, 2004b); (IARC, 2012).
Health outcomes considered to have *slight* evidence		
Prostate cancer	Tier 2	**Slight**. Based on systematic review conducted by EPA, which is similar to associations noted in (IARC, 2004b); (IARC, 2012).
Pancreatic cancer	Tier 3	**Slight**. Based on systematic review conducted by EPA and associations noted in (IARC, 2004b).
Renal disease	Tier 3	**Slight**. Based on systematic review conducted by EPA.

[a]In cases of Tier 2 or 3 health outcomes, the results and conclusions of systematic reviews conducted by EPA formed the primary rationale for identifying a health outcome as having robust, moderate, or slight strength of evidence. For health outcomes that also had meta-analyses conducted by outside groups, the meta-analyses are considered supplemental information. Relevant primary studies included in the meta-analyses were considered in the systematic reviews conducted by EPA.

[b]These outcomes considered along with the larger ischemic heart disease database; the strength of the epidemiologic database was based on the full set of all studies for all endpoints.

2.3.2. Mode-of-Action Analyses

EPA Cancer Guidelines (U.S. EPA, 2005a) discuss the use of an MOA framework as an analytic tool to evaluate the mechanistic evidence for carcinogenicity or any toxicity within hypothesized MOAs. The Cancer Guidelines state that such analyses are used "to address the question of human relevance of animal tumor responses; to address differences in anticipated response among humans, such as between children and adults or men and women; and as the basis of decisions about the anticipated shape of the dose response relationship" (U.S. EPA, 2005a).

The EPA Cancer Guideline recommendations for MOA analyses are typically applied for chemicals for which human evidence is insufficient or human relevance needs to be established. Inorganic arsenic, a known human carcinogen, is a chemical with a large amount of epidemiological evidence of carcinogenesis resulting from exposure. The carcinogenic risk to humans has been established by numerous government agencies, including the World Health Organization (WHO) International Agency for Research on Cancer (IARC), which identifies inorganic arsenic as a Group 1 carcinogen: "a compound carcinogenic to humans" (IARC, 2012). With respect to the Cancer Guidelines and (NRC, 2013) recommendations regarding interhuman variability, extensive information on risk modifiers in numerous epidemiological studies of inorganic arsenic is available. Hence, a MOA analysis addressing potential differences in response across human populations were not considered essential as it is expected that such analyses can be conducted using information from the available epidemiological studies.

With respect to using MOA and mechanistic data to inform dose-response, EPA conducted a significant number of analyses of mechanistic information (see Appendix A), and a case study using idiopathic bladder cancer was undertaken to address the feasibility of using this information to inform dose–response modeling with respect to the shape of the curve, particularly in the low-dose region. Idiopathic bladder cancer was selected for the case study given the abundance of mechanistic data available for use in conducting the MOA analysis. The results of the literature review presented in Appendix A and case study were interpreted in the context of whether these analyses provided a firmer basis for reaching conclusions about the shape of the dose-response curve in the low-dose region compared with using the multiple epidemiological studies available that directly assess the effects of low-dose arsenic exposures in various U.S. populations. Ultimately, the MOA analyses were not considered more suitable than the epidemiological studies. This reliance on the epidemiological studies for use in dose-response analysis is similar to the recent meta-regression analysis of arsenic epidemiology studies conducted by (TCEQ, 2017) and (Lynch et al., 2017b); (Lynch et al., 2017a),and is consistent with the focus on epidemiology studies in earlier assessments (EFSA, 2009);(RIVM, 2001);(FDA, 2005);(Health Canada, 2006);(NIOSH, 2005);(OSHA, 2005);(McGeer et al., 2004);(U.S. EPA, 2007);(U.S. EPA, 2002a);(ATSDR, 2007);(IARC, 2004a);(OEHHA, 2014).

The major challenge in using MOA analyses to reach conclusions about the shape of the dose-response relationship is that mechanisms of arsenic-associated disease induction are complex,

inter-related, differentially applicable to cancer and noncancer outcomes under consideration, and likely interoperable in different ways across the concentration ranges tested. There is little evidence that directly addresses this complexity in the low-dose region. Moreover, much of the primary evidence is based on in vitro studies conducted at high concentrations (see Appendix A), raising concerns about applicability to low-dose effects. In other cases, mechanistic evidence comes from rodent studies; these animals are generally considered less susceptible to inorganic arsenic than humans. The MOA analysis for bladder cancer supported findings from epidemiological studies on risk modifiers (i.e., smoking, genetic polymorphisms, methylation capacity) that may affect arsenic associated bladder cancer risk. However, while the MOA evaluation provided additional support by identifying arsenic-specific mechanisms and risk modifiers likely to increase the risk of human bladder cancer, it is uncertain how this information might be used to inform the quantitative dose-response analysis. Conducting a similar analysis for other prioritized outcomes is hindered by the lack of a complete MOA for any health outcome and the likelihood that most, if not all, health outcomes associated with arsenic exposure involve multiple interactive MOAs. These challenges have been long recognized, and the NRC acknowledged uncertainty about whether such analyses would be feasible without further research (NRC, 2013).

The determination to not use animal-based MOA analyses in the dose-response analysis is further supported by both the abundance of epidemiological studies of low-level exposures to arsenic and the increased power and confidence in low-dose extrapolations afforded by new developments in Bayesian meta-regression methods that combine data from multiple studies into a single analysis. The hierarchical Bayesian method allows for the analysis of case-control and cohort studies, as well as low- and high-dose studies, simultaneously. In addition, this approach makes no assumption on the shape of the dose-response curve (i.e., linear vs. nonlinear) or whether a threshold exists, except that it does not allow for a change in the dose-response direction (e.g., a "J"-shaped dose-response curve). In selecting studies for inclusion in these meta-analyses, priority will be given to studies with well-characterized exposures during all life stages, including early life (e.g., pregnancy). Finally, Bayesian meta-regression methods are in line with the 2005 EPA Cancer Guidelines, which recommends that "when several studies are available for dose-response analysis, meta-analysis can provide a systematic approach to weighing positive studies and those studies that do not show positive results and calculating an overall risk estimate with greater precision." Additional details on the Bayesian meta-regression analysis are summarized below in Section 5.

The approach for dose-response analysis in the iAs assessment is consistent with the 2005 EPA Cancer Guidelines two-step approach to distinguish the dose-response data from inferences made about lower doses: the first step involves analyses in the range of observations made in the experimental and epidemiological studies and the second step involves extrapolation into the lower dose range, taking into consideration what is known about the agents' MOA (U.S. EPA, 2005a). However, for iAs, the second extrapolation step is not needed because EPA is modeling human data,

and the lower range of exposures reported in the epidemiological studies are very near U.S. background exposure levels.

3. SYSTEMATIC REVIEW METHODS USED TO PRIORITIZE HEALTH OUTCOMES

3.1. SPECIFIC AIMS

- Identified epidemiological (i.e., human) studies reporting effects of exposure to inorganic arsenic, focusing on the health outcomes suggested by the National Research Council (NRC, 2013):
    - Tier 1: Bladder cancer, lung cancer, skin cancer, skin lesions, ischemic heart disease.
    - Tier 2: Diabetes, birth weight, neurodevelopmental effects, immune effects, renal cancer, prostate cancer, nonmalignant respiratory disease.
    - Tier 3: Hypertension, stroke, fetal loss/stillbirth/neonatal mortality, liver cancer, pancreatic cancer, renal disease.
- Conducted study evaluations (risk of bias) for individual studies according to the National Toxicology Program (NTP) Office of Health Assessment and Translation (OHAT) approach (NTP, 2013), with some assessment -specific clarifications. Studies classified as low quality or uninformative were not considered further for dose -response analysis.
- Extracted data on relevant health outcomes from epidemiological studies.
- For each health outcome specified above, expressed strength-of-evidence synthesis conclusions across epidemiology studies (or subsets of studies) by relying on conclusions from other assessments, conducting new systematic review evidence synthesis analysis, or by a combination of both.
    - Because bladder, lung, and skin cancer are accepted hazards of inorganic arsenic exposure (NTP, 2016);(ATSDR, 2007);(ATSDR, 2016);(IARC, 2004b);(IARC, 2012);(WHO, 2011b);(WHO, 2011a);(Lynch et al., 2017b);(Lynch et al., 2017a), the strength of evidence for these health outcomes was considered *robust*, and no new evidence synthesis was conducted by EPA. The assessment will focus on studies for these outcomes considered suitable for dose-response analysis.
    - For the other health outcomes listed above, a new systematic review evidence synthesis analysis was conducted to characterize the strength of evidence for potential hazard.

3.2. POPULATIONS, EXPOSURES, COMPARATORS, AND OUTCOMES (PECO)

A populations, exposures, comparators, and outcomes (PECO; see Table 3-1) was used as an aid to focus the research question(s) search terms and to guide study inclusion/exclusion criteria during literature screening. Changes in the PECO over time are reflected in the Table 3-1. The PECO for inorganic arsenic was based on a review of the evidence and recommendations presented in the 2013 National Research Council *Critical Aspects of EPA's Integrated Risk Information System Assessment of Inorganic Arsenic* (NRC, 2013) and focused on epidemiological evidence only.

The PECO criteria used to identify relevant studies evolved over time to reflect problem formulation activities, including NRC consultations, that narrowed the focus of the assessment.

- 2012–2013 screening: Broad problem formulation screening to include tracking of epidemiological, animal, and mechanistic evidence with no restriction on type of health outcome (both cancer and noncancer).

- Post-2013 screening: Based on the 2013 NRC consultation, screening efforts focused on outcomes classified by the NRC as Tier 1, 2, or 3 (cancers of the bladder, lung, skin, kidney, liver, prostate, pancreas; skin lesions; or noncancer effects of the circulatory system; pregnancy and birth outcomes; neurodevelopmental effects; diabetes; immune system; respiratory disease [nonmalignant]; or renal disease).

- Post-2017 screening: Based on the post-2013 problem formulation activities, screening efforts have focused on health outcomes with *robust* or *moderate* evidence (see Table 2-2). These outcomes include cancers of the bladder, lung, kidney, liver, and skin; and noncancer effects of inorganic arsenic on the circulatory system (ischemic heart disease, hypertension, and stroke), reproductive system (including pregnancy and birth outcomes), developmental outcomes (including neurodevelopmental toxicity), endocrine system (including diabetes), immune system, respiratory system, and skin. The broad search strategy presented in Section 3.3 was refined to focus on human studies and outcomes of interest by using a filter available in SWIFT-Review software (filtered by health outcomes and human evidence stream). Screening for relevance was then conducted in SWIFT-Active software. Any animal or mechanistic study identified using this narrowed search strategy was tagged as supplemental material.

- Post-2019 screening: Since oral exposure is the primary route of exposure for the general population, inhalation exposure to inorganic arsenic was not evaluated further. In addition, the primary agency scoping need is oral, inhalation studies are mainly occupational studies, and the bulk of new epidemiological studies concern oral exposures. Further prioritization of health outcomes based on hazard judgment, RRB, and potential use for benefit-costs analysis by program offices. These outcomes include bladder cancer, lung cancer, DCS, diabetes, pregnancy outcomes, and neurodevelopmental effects.

Table 3-1. Populations, exposures, comparators, and outcomes (PECO)

PECO element	Evidence
Populations	**2012–2013:** Focused on human studies and considered animal and mechanistic studies (U.S. EPA, 2014). Animal studies may provide supporting evidence for hazard identification. If health effects are reported exclusively in animal studies, mechanistic data will be used to determine human relevance of these effects. Animal and mechanistic studies may also inform susceptibility and dose-response. Post-2013: Focused on human studies and considered animal and mechanistic studies (U.S. EPA, 2015). Animal studies may provide supporting evidence for hazard identification. Animal and AOPn information may also inform susceptibility and dose-response. **Post-2017 (current):** Focuses on human studies only to include any population and life stage (occupational or general population, including children and other sensitive life stages or populations).
Exposures	Subchronic- or chronic-duration studies of interest provide quantitative estimates of exposure with measurements based on biomonitoring data (e.g., hair, nails, urine, or blood), inhalation (air exposures [µg/m^3]), drinking water exposures (µg/L), cumulative exposures (µg/m^3-yr; µg/L/-yr), and doses expressed as µg/d and µg/kg-d. Studies with episodic or acute exposures will be excluded (i.e., poisonings or other short-term exposures that last up to 30 d). Studies using arsenicals, primarily arsenic trioxide and Fowler's solution will be excluded because chemotherapeutic agents are not within the scope of this review. Studies using arsenide (As^{3-}), an inorganic form of arsenic, also will be excluded. Exposures usually occur via the gas arsine and result in a different, distinctive toxicological profile based on binding to hemoglobin and red blood cell lysis. **Post-2019 (current)** screening to focus on oral exposure.
Comparators	A comparison or referent population exposed to lower levels (or no exposure/exposure below detection limits) of inorganic arsenic, or exposure to inorganic arsenic for shorter periods of time, or cases vs. controls. Exposure-response quantitative results are presented in sufficient detail (e.g., odds ratios or relative risks with associated confidence intervals, numbers of cases/controls, etc.).
Outcomes	**2012–2013** broad problem formulation screening: All health outcomes (both cancer and noncancer) (U.S. EPA, 2014). Post-2013 screening to focus on outcomes classified by the NRC as Tier 1, 2, or 3: Cancers of the bladder, lung, skin, kidney, liver, prostate, pancreas; skin lesions; or noncancer effects of the circulatory system; pregnancy and birth outcomes; neurodevelopmental effects; diabetes; immune system; respiratory disease (nonmalignant); or renal disease (U.S. EPA, 1995). **Post-2017** screening of health outcomes prioritized for inclusion in the assessment: cancers of the bladder, lung, kidney, liver, and skin; noncancer effect of inorganic arsenic on the circulatory system (ischemic heart disease, hypertension, and stroke), reproductive system (including pregnancy and birth outcomes), developmental outcomes (including neurodevelopmental toxicity), endocrine system (including diabetes), immune system, respiratory system, and skin. **Post-2019 (current)** screening to further prioritize health outcomes based on hazard judgement, RRB, and potential use for benefit-costs analysis by program offices: bladder cancer, lung cancer, DCS, diabetes, pregnancy outcomes, and neurodevelopmental effects. Note: A broad outcome search strategy was retained during the different phases of outcome prioritization. Epidemiological studies on other health outcomes not prioritized are tagged during screening to monitor for new studies that may affect the problem formulation decisions described above.
PBPK models	Studies describing PBPK models for inorganic arsenic will be included. Studies describing quantitative models or data for understanding kinetics in biological media will be tracked as "potentially relevant supplemental material."

AOPn = Adverse Outcome Pathway network; PBPK = physiologically based pharmacokinetic.
Note: Post-2017 (current) Animal and mechanistic data are considered supplemental material and not tracked as PECO relevant.

3.3. LITERATURE SEARCH STRATEGIES

Literature search strategies were originally developed using keywords related to identifying relevant forms of arsenic, without the restriction of type of evidence (human, animal, mechanistic) or type of health outcome. Development of the search strategy for each topic area was conducted by identifying relevant search terms by (1) reviewing PubMed's Medical Subject Headings (MeSH) for relevant and appropriate terms, (2) extracting key terminology from relevant reviews and a set of previously identified primary data studies that are known to be relevant to the topic ("test set"), and (3) reviewing search strategies presented in other reviews. Broad search terms were used to collect references from PubMed, Web of Science, and Toxline. The search strategy was run, and the results were assessed to ensure that previously identified relevant primary studies were retrieved. Because each database has its own search architecture, the resulting search strategy was tailored to account for each database's unique search functionality.

Searches were not restricted by publication date or language. Literature searching was conducted by EPA's Health and Environmental Research Online (HERO) staff and stored in the HERO database.[1] The literature search will be updated during the assessment to identify literature published during the review. The last literature search update will occur within a year before the planned release of the draft document for public comment and peer review.

The IRIS Program takes extra steps to ensure identification of pertinent studies by encouraging the scientific community and the public to identify additional studies and ongoing research; by searching for data submitted under the Toxic Substances Control Act or the Federal Insecticide, Fungicide, and Rodenticide Act; and by considering recent studies that would impact the credibility of the conclusions, even during the review process.[2] Studies identified after peer review begins will only be considered for inclusion if they are directly applicable to the PECO eligibility criteria and fundamentally alter the assessment's conclusions.

3.4. USE OF MACHINE LEARNING TO PRIORITIZE STUDIES FOR SCREENING

Following the original literature search in December 2012, the references were clustered into groups based on language similarity (i.e., natural language processing) using OmniViz reference visualization software (Instem, Staffordshire, United Kingdom). This supervised clustering methodology is further described in (Varghese et al., 2017). The initial literature search was designed to be comprehensive and not miss potentially relevant studies; clustering helped to more efficiently identify those references most likely to contain data relevant to hazard identification. Approximately 900 additional references were used as "seeds"; these "seed" references are studies (both human and animal) previously identified as relevant to hazard

[1]Health and Environmental Research Online: https://hero.epa.gov/hero/.
[2]IRIS "stopping rules": https://www.epa.gov/sites/production/files/2014-06/documents/iris_stoppingrules.pdf.

identification in peer-reviewed arsenic human health risk assessments published by government agencies (IARC, 2012); (ATSDR, 2007); (Health Canada, 2006); (IARC, 2004b); (NRC, 1999). The "seeds" and literature search results were combined, and the titles and abstracts of the references were grouped based on similarity using natural language processing. Reference clusters containing one or more of the "seed" references were selected to create the health effects cluster of 3,715 references that were then manually screened for relevance. These 3,715 references moved through the steps described in the following sections to determine their relevance to hazard identification. References identified after the initial 2012 literature search were screened manually, and computerized clustering was not applied. The last literature search update was conducted in August 2022.

Studies identified in the most recent literature search update will undergo screening for PECO relevance. Studies on health outcomes that EPA has characterized the strength of the evidence base to be *robust* will not undergo risk of bias evaluation unless it is determined that the hazard conclusion will change or if a study can be used in dose-response analyses. A screening and survey of the studies will be conducted to determine if the new studies will impact existing hazard conclusion. Studies on health outcomes that EPA has characterized the strength of the evidence base to be *moderate* will undergo risk of bias evaluation to determine if new studies will change the hazard conclusion and/or impact dose-response analyses. To further screen studies for dose-response utility, additional consideration will be given to study type and key confounding factors, such as smoking, that are important to the dose-response approach. The characterization of newly identified studies will focus on EPA's judgment of whether the studies would have a material impact on the conclusions (i.e., identified hazards or toxicity values) in the external review draft.

As mentioned above, the PECO criteria used to identify relevant studies evolved to reflect problem formulation activities, including NRC consultations, that narrowed the focus of the assessment. The broad outcome search strategy was retained during the different phases of outcome prioritization. Epidemiological studies on other health outcomes not prioritized were tagged during screening to monitor for new studies that may affect the problem formulation decisions described above.

3.5. NON-PEER-REVIEWED DATA

IRIS assessments rely mainly on publicly accessible, peer-reviewed studies. However, it is possible that gray literature (i.e., not reported in the peer-reviewed literature) directly relevant to the PECO may be identified (e.g., dissertations, etc.) during assessment development. Should such studies substantially affect assessment decisions or conclusions (i.e., potential to affect PECO statement, hazard conclusions, or dose-response analysis), the EPA can obtain external peer review if the owners of the data are willing to have the study details and results made publicly accessible. This independent, contractor-driven peer review would include an evaluation of the study like that done for a peer-reviewed journal article. The contractor would identify and select two to three

scientists knowledgeable in scientific disciplines relevant to the topic as potential peer reviewers. Those selected would be screened for conflict of interest prior to confirming their service. In most instances, the peer review would be conducted by letter. The study authors would be informed of the outcome of the peer review and given an opportunity to clarify issues or provide missing details. The study and its related information, if used in the IRIS assessment, would become publicly available. In the assessment, EPA would acknowledge that the document underwent external peer review managed by the EPA, and the names of the peer reviewers would be identified.

Unpublished (e.g., raw) data from personal author communication can supplement a peer-reviewed study if the information is made publicly available (typically through documentation in HERO).

3.6. SCREENING PROCESS

The 3,715 studies identified from reference clustering and the additional references identified after the initial 2012 literature search were manually screened for applicability to PECO. Studies that comply with the criteria specified in the PECO (see Table 3-1) are considered eligible for inclusion, while those that do not meet these criteria will be excluded. In addition to these criteria, the exclusion criteria noted below are applied.

- Records that do not contain original data, such as scientific literature reviews, editorials, or commentaries. Although not considered PECO relevant, these studies are tracked during screening as potentially relevant supplemental materials.
- Records considered potentially relevant supplemental materials. Although not directly considered PECO relevant, these studies are tracked during the screening process as described below.
- Non-peer-reviewed studies with original data (e.g., abstracts, posters, dissertations).
- Retracted studies.

References were moved through the steps described below to determine their relevance to hazard identification. Following a pilot phase to calibrate screening guidance, two screeners independently conducted a title and abstract screen of the search results using a structured form in DRAGON[3] online to identify records that appeared to meet the PECO eligibility criteria. Records that were not excluded based on the title and abstract advanced to full-text review. Screening conflicts were resolved by discussion among the primary screeners with consultation by a third reviewer or technical advisor (if needed) to resolve any remaining disagreements. Assessment of

[3]DRAGON was an online tool for systematic review developed by ICF. DRAGON stored qualitative and quantitative data for purposes of problem formulation, literature screening, risk-of-bias evaluation, and data integration. DRAGON is no longer available, and data has been moved into HAWC.

eligibility status of non-English studies was facilitated using Google Translate for abstracts and, if needed, native-language speakers at the EPA.

Many informative studies important to consider in the assessment do not meet the PECO but need to be tracked during screening as potentially relevant to the research question(s). Such studies can include information on ADME; exposure characteristics; population demographics; nonmammalian model systems; human or animal cells, tissues, or biochemical reactions with in vitro exposure regimens; bioinformatics pathways of disease analysis; or high-throughput screening data. These studies will be categorized (i.e., tagged) during the title and abstract screening process as "potentially relevant supplemental material."

Records that are not excluded based on the title and abstract advanced to full-text review. Full-text copies of potentially relevant records identified from title and abstract screening are retrieved, stored in the HERO database, and independently assessed by two screeners to confirm eligibility according to the PECO criteria. Screening conflicts are resolved by discussion among the primary screeners with consultation by a third reviewer or technical advisor (as needed) to resolve any remaining disagreements.

The included and excluded studies are posted on the project page for this assessment in the HERO database http://hero.epa.gov/index.cfm/project/page/project_id/2211.

3.6.1. Multiple Publications of the Same Data

When multiple publications use the same or overlapping data, all publications on the research will be included, with one selected as the primary study; the others will be considered as secondary publications with an annotation indicating their relationship to the primary record during data extraction. For epidemiology studies, the primary publication will generally be the one with the longest follow-up, the largest number of cases, or the most recent publication date. EPA will include relevant data from all publications of the study, but if the same outcome is reported in more than one report, the data will only be extracted once.

3.7. LITERATURE SURVEYS AND SUMMARY-LEVEL INVENTORIES

During manual title/abstract and full-text screening, studies were categorized (or "tagged") based on the following categories to help organize the literature, including both studies meeting the PECO-based inclusion criteria and potentially relevant supplemental materials. Summary-level inventories of basic study information (e.g., species; health outcomes) have been developed to aid subsequent steps, including study evaluations of included studies (U.S. EPA, 2014).

- "Included": Epidemiological studies or physiologically based pharmacokinetic (PBPK) models meeting PECO-based inclusion criteria.
- "Potentially relevant supplemental materials":
  - Epidemiological studies on other health outcomes not listed in PECO.

- Toxicology: Experimental animal studies presenting original data potentially supportive of assessment of chronic exposure to inorganic arsenic (iAs).

- Mode of action/mechanistic: Studies examining the molecular and/or cellular events and alterations in system biology occurring after iAs exposure (e.g., alterations in epigenomics, genomics, oxidative stress, immune function, and endocrine disruption). Metabolites of iAs are only considered as they pertain to MOA. Bioassays of metabolites may be cited if they inform the MOA.

- Meta-analyses that contain original analyses.

- Susceptibility: Studies that do not meet PECO-based inclusion criteria, but which include analyses of health effects relevant to the PECO that are evaluated based on potential risk modifiers (e.g., smoking, genetic polymorphisms, susceptibility due to methylation capacity, socioeconomic factors, ethnicity).

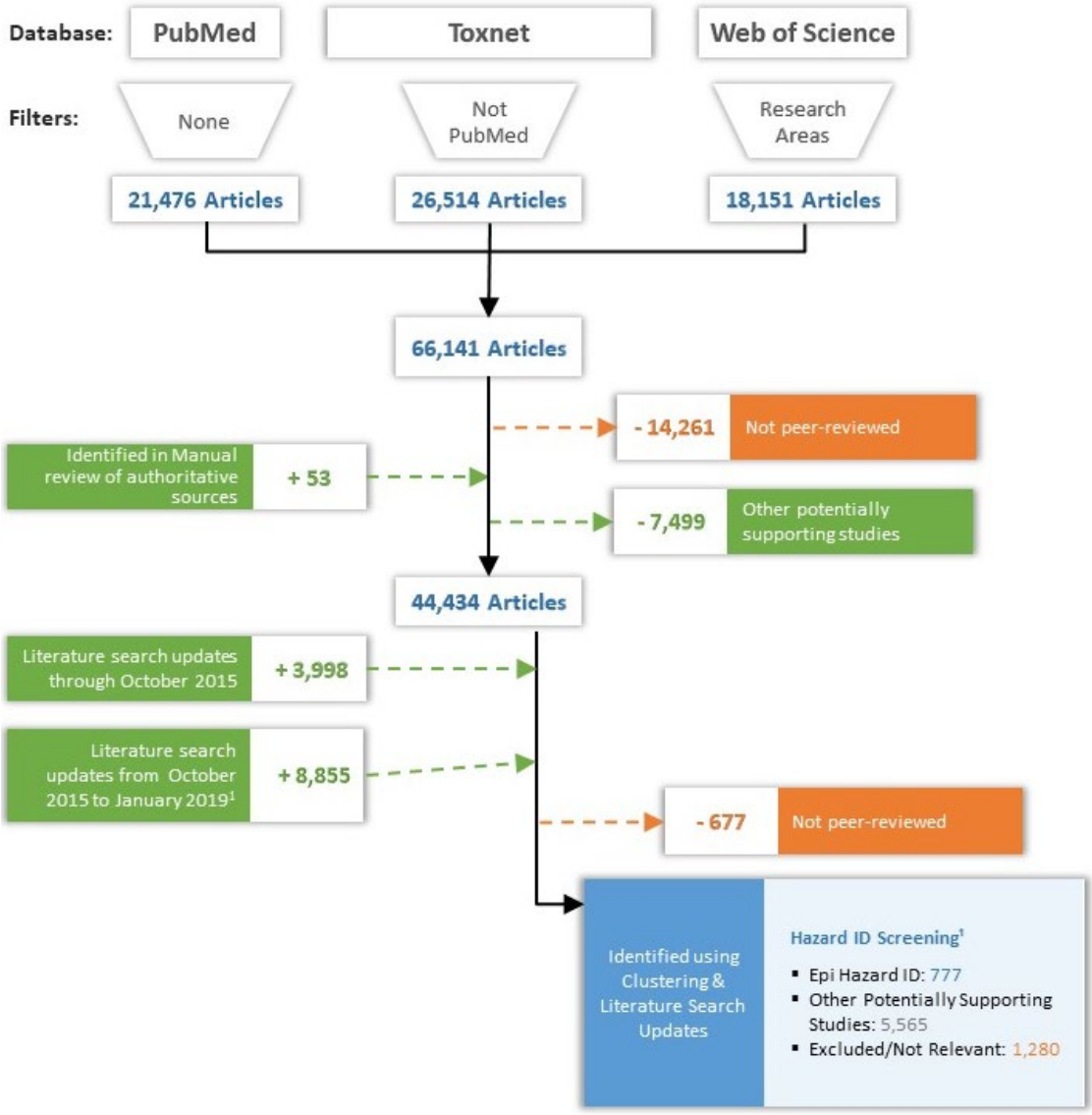
- ADME/toxicokinetics (TK): Studies that examine internal dose metrics, absorption, distribution, metabolism, and excretion (i.e., TK).

- Exposure assessment: Studies that describe exposure to arsenic in the air, water, food, or through dermal contact. Includes bioavailability studies for the different media and studies that measured arsenic levels in humans (e.g., in nails, urine, blood) and studies that do not evaluate health outcomes but provide an understanding of arsenic exposures associated with health effects.

- Life stages: Epidemiological and experimental animal studies help characterize in utero, childhood, puberty, pregnancy, women of child-bearing age, old-age susceptibilities.

3.8. TRACKING STUDY ELIGIBILITY AND REPORTING THE FLOW OF INFORMATION

The literature search and screening process is summarized in the study flow diagram (see Figure 3-1) and will be updated in HERO. Categories for exclusion include the following: (1) not relevant to PECO; (2) is a review, commentary, or letter with no original data (with exception of meta-analyses); (3) is a conference abstract or thesis (and the criteria for including unpublished data, described above, are not met); or (4) unable to obtain full text.



[1] Screening of literature search updates from October 2015 to January 2019 is underway and will be updated in HERO.

Figure 3-1. Literature search and screening process for inorganic arsenic assessment.

3.9. STUDY EVALUATION (REPORTING, RISK OF BIAS, AND SENSITIVITY) STRATEGY FOR EPIDEMIOLOGICAL STUDIES

Epidemiologic studies containing exposure- or dose-response data were subject to risk-of-bias (RoB) evaluations to assess aspects of internal validity of study findings based on study design and conduct for hazard identification. Key concerns are potential bias (factors that affect the magnitude or direction of an effect) and insensitivity (factors that limit the ability of a study to detect a true effect). Risk of bias for each study was evaluated using questions across seven evaluation domains (i.e., selection, confounding, performance, attrition, detection, selective reporting bias, and other) adapted from the OHAT approach (NTP, 2013)[4] (see Table 3-2). Risk of bias was assessed for each study question using a rating system with four categories as follows: low bias, probably low bias, probably high bias, and high bias (see Table 3-3). Evaluations were documented using DRAGON online at the health-outcome level. Each study was evaluated independently by two scientists who used the draft OHAT approach for systematic review (NTP, 2013) and arsenic-specific clarifications developed, as needed, in consultation with technical experts for evaluation questions (see Appendix C). The supporting rationale for each rating was documented by the reviewers. After independently reviewing a study, the two reviewers discussed differences and resolved discrepancies between their ratings and rationales.

[4]The OHAT method was used for this assessment because the current approach being used in IRIS had not been fully developed at the time these study evaluations were being conducted (2012 to 2017).

Table 3-2. Risk of bias considerations

Category	Risk-of-bias question
Selection	1) Was administered dose or exposure level adequately randomized?[a]
	2) Was allocation to study groups adequately concealed?[a]
	3) Were the comparison groups appropriate?
Confounding	4) Did the study design or analysis account for important confounding and modifying variables?
	5) Did researchers adjust or control for other exposures that are anticipated to bias results?
Performance	6) Were experimental conditions identical across study groups?[a]
	7) Did researchers adhere to the protocol?
	8) Were the research personnel and human subjects blinded to the study group during the study?[a]
Attrition	9) Were outcome data complete without attrition or exclusion from analysis?
Detection	10) Were the outcome assessors blinded to the study group or exposure level?
	11) Were confounding variables assessed consistently across groups using valid and reliable measures?
	12) Can we be confident in the exposure characterization?
	13) Can we be confident in the outcome assessment?
Selective reporting bias	14) Were all measured outcomes reported?
Other	15) Were there no other potential threats to internal validity (e.g., statistical methods were appropriate)?

[a]These questions were not relevant for evaluating observational studies.

Table 3-3. Risk of bias ratings

Risk-of-bias rating	Description
(++) Definitely low	There is direct evidence of low risk-of-bias practices (direct evidence is an explicit statement(s), generally in the study report or through contacting the authors).
(+) Probably low	There is indirect evidence of low risk-of-bias practices, or it is deemed by the risk-of-bias evaluator that deviations from low risk-of-bias practices for these criteria during the study would not appreciably bias results, including consideration of direction and magnitude of bias (indirect evidence provides information to address the risk-of-bias question but falls short of direct evidence).
(−) Probably high	There is indirect evidence of high risk-of-bias practices, or there is insufficient information provided about relevant risk-of-bias practices.
(− −) Definitely high	There is direct evidence of high risk-of-bias practices (could include specific examples of relevant high risk-of-bias practices).

The OHAT risk-of-bias tool conclusions were combined with conclusions about sensitivity for each study to arrive at a conclusion about study confidence, which then was incorporated in the IRIS framework for evidence integration. The OHAT risk-of-bias tool conclusions were considered along with the identified strengths and limitations to reach a study confidence classification of *high*, *medium*, or *low* confidence, or *uninformative* for a specific health outcome. This classification was based on the reviewer judgments across the evaluation domains and considered the likely effect any noted deficiencies in bias and sensitivity or inadequate reporting would have on the results. The classifications, which reflected a consensus judgment between reviewers, are defined as follows:

- *High* confidence: No notable deficiencies or concerns were identified; the potential for bias is unlikely or minimal, and the study used sensitive methodology. *High*-confidence studies generally reflect judgments of definitely low risk of bias across all or most evaluation domains.

- *Medium* confidence: Possible deficiencies or concerns were noted, but the limitations are unlikely to be of a notable degree. Generally, *medium*-confidence studies include low or probably low risk of bias across most domains, with the effect of any identified limitation not being judged as severe.

- *Low* confidence: Deficiencies or concerns are noted, and the potential for bias or inadequate sensitivity could have a significant impact on the study results or their interpretation. Typically, *low*-confidence studies have a high risk-of-bias evaluation for one or more domains, although some *medium*-confidence studies may have a high rating in the domain(s) considered to have less influence on the magnitude or direction of effect estimates. Generally, *low*-confidence results are given less weight than *high*- or *medium*-confidence results during evidence synthesis and integration and are generally not used for either hazard identification or dose-response unless they are the only studies available. Studies rated as *low* confidence only because of sensitivity concerns about bias towards the null will be asterisked or otherwise noted because they may require additional

consideration during evidence synthesis. Observing an effect in these studies may increase confidence during evidence synthesis, assuming the study is otherwise well conducted.

- *Uninformative*: Serious flaw(s) makes the study results unusable for informing hazard identification. Studies with definitely high risk-of-bias judgements in any evaluation domain are almost always classified as *uninformative* (see explanation above). Studies with multiple probably high risk-of-bias judgments across domains may also be considered *uninformative*. *Uninformative* studies will not be considered further in the synthesis and integration of evidence.

3.10. DATA EXTRACTION

Data extraction and content management was carried out using DRAGON (see Section3.3 for a list of data abstraction elements) and has been migrated to HAWC. Data abstraction was performed by one evaluation team member and checked by one to two other members.

In selecting specific epidemiological study results and data to present in the evidence table, adjusted statistical estimates (e.g., odds ratios adjusted for confounding factors) were presented rather than unadjusted or raw estimates, when possible. Data for all exposure metrics (including water, hair, nails, urine) are presented in the evidence tables. When multiple measures were presented for the same exposure metric, cumulative arsenic exposure levels were selected for inclusion in the evidence tables, when available. Total urinary arsenic levels were selected over concentrations of individual metabolites, when available. All results were included, regardless of statistical significance.

Routine attempts were made to obtain missing information from epidemiologic studies, focusing on information required to conduct a meta-analysis. Outreach to study authors was considered unsuccessful if researchers did not respond to an email or phone request within 1 month of the attempt to contact.

All studies identified as potentially relevant for a specific health outcome were included in the evidence tables regardless of the results (positive, negative, or null). Evidence tables include information for comparing key features like study design, exposure metrics, and dose-response information. The data presented in the evidence table focus on general population risks. If the study only reported on a susceptible population (e.g., smokers) or life stage (e.g., childhood), these data are presented in the evidence tables and noted accordingly. These tables provide an overview of the key findings in a study and do not necessarily include all data or results presented in a study.

3.11. EVIDENCE SYNTHESIS OF EPIDEMIOLOGICAL EVIDENCE

Each synthesis is written to provide a summary discussion of the available evidence that addresses considerations that may suggest causation adapted from considerations for causality introduced by Austin Bradford Hill (Hill, 1965), including consistency, exposure-response relationship, strength of the association, temporal relationship, biological plausibility, coherence, and "natural experiments" in humans (U.S. EPA, 1994); (U.S. EPA, 2005a) (see Table 3-4). The

approach taken for evidence synthesis within the IRIS Program is informed by both Hill and another widely used approach, the Grading of Recommendations Assessment, Development and Evaluation (GRADE) framework, which includes consideration of many of the concepts but provides more details on how to evaluate and document the expert judgments embedded in the process of evidence synthesis (Guyatt et al., 2011); (Schünemann et al., 2011). Importantly, the approach to the process of evidence synthesis explicitly considers and incorporates the conclusions from the individual study evaluations.

As indicated earlier, skin., bladder, and lung cancer and skin lesions are accepted hazard outcomes for inorganic arsenic (ATSDR, 2007); (Health Canada, 2006); (IARC, 2004b); (IARC, 2012); (NRC, 2013) and are considered as *robust* evidence. Evidence synthesis conclusions are developed for noncancer effects of the circulatory system, pregnancy and birth outcomes, neurodevelopmental effects, and diabetes, as described below (see Table 3-4).

Table 3-4. Summary of health outcomes for new evidence synthesis and dose-response analyses

Hazards prioritized based on HI judgments of Robust or moderate	EPA's Hazard judgment	NRC Tier (NRC, 2013)	EPA's RRB analysis eliminated	Indicated for dose-response to (NASEM, 2019) basis: hazard judgement, RRB, and benefit-cost	Accepted causal, do not need synthesis	Evidence synthesis	Modeled in assessment
lung cancer	robust	1		x	X		X
bladder cancer	robust	1		x	X		X
skin cancer	robust	1	X (analysis post-NAS review)	x	X		No, RRB comparison post-NAS
DCS	robust	1		x		X	X
skin lesions	robust	1	X (analysis post-NAS review)	x	X		No, RRB comparison post-NAS
diabetes	robust	2		X (benefit-cost)		X	X (benefit-cost)
pregnancy outcomes	moderate	2		X (benefit-cost)		X	X (benefit-cost)
renal cancer	moderate	2		x			No, moderate and no benefit-cost need
nonmalignant respiratory	moderate	2		x			No, moderate

Hazards prioritized based on HI judgments of Robust or moderate	EPA's Hazard judgment	NRC Tier (NRC, 2013)	EPA's RRB analysis eliminated	Indicated for dose-response to (NASEM, 2019) basis: hazard judgement, RRB, and benefit-cost	Accepted causal, do not need synthesis	Evidence synthesis	Modeled in assessment
							and no benefit-cost need
neurodevelopmental	moderate	2		X (benefit-cost)		X	X (benefit-cost)
immune	moderate	2	X				No, RRB
liver cancer	moderate	3		x			No, moderate and no benefit-cost need

Table 3-5. Information most relevant to describing primary considerations informing causality during evidence syntheses

Consideration	Description and synthesis methods
Consistency	Examines the similarity of results (e.g., direction; magnitude) across studies. When inconsistencies exist, the synthesis considers whether results were "conflicting" (i.e., unexplained positive and negative results in similarly exposed human populations) or "differing" (i.e., mixed results explained by differences between human populations, exposure conditions, or study methods) (U.S. EPA, 2005a) based on analyses of potentially important explanatory factors, for example, review of results across: • Confidence in studies' results, including study sensitivity (e.g., some study results that appear to be inconsistent may be explained by potential biases or other attributes that affect sensitivity, resulting in variations in the degree of confidence accorded to the study results); • Exposure, including route (if applicable), levels, duration, etc. • Populations or species, including consideration of potential susceptible groups or differences across life stage at exposure or endpoint assessment; and • Toxicokinetic information as an explanation for any observed differences in responses across routes of exposure, other aspects of exposure, species, or life stages. The interpretation of the consistency of the evidence and the magnitude of the reported effects will emphasize biological significance as more relevant to the assessment than statistical significance. Statistical significance (as reported by *p*-values, etc.) provides no evidence about effect size or biological significance, and a lack of statistical significance will not automatically be interpreted as evidence of no effect.

Consideration	Description and synthesis methods
Strength (effect magnitude) and precision	Examines the effect magnitude or relative risk, based on what is known about the assessed endpoint(s), and considers the precision of the reported results based on analyses of variability (e.g., confidence intervals; standard error). Syntheses will analyze results both within and across studies and may consider the utility of combined analyses (e.g., meta-analysis). While larger effect magnitudes and precision (e.g., $p < 0.05$) help reduce concerns about chance, bias, or other factors as explanatory, syntheses should also consider the biological or population-level significance of small effect sizes. Thus, a lack of statistical significance should not be automatically interpreted as evidence of no effect.
Biological gradient/dose-response	Examines whether the results (e.g., response magnitude; incidence; severity) change in a manner consistent with changes in exposure (e.g., level; duration). Syntheses will consider relationships both within and across studies, acknowledging that the dose-response curve (e.g., shape) can vary depending on the outcome and the toxicokinetic of the chemical (among other things). Evidence of a monotonic dose-response relationship often strengthens evidence synthesis conclusions, although there are cases in which monotonicity should not necessarily be expected (e.g., different outcomes may be expected at low vs. high doses due to activation of different mechanistic pathways or induction of systemic toxicity at very high doses). For reversible responses, decreases in a response after cessation of exposure also may strengthen synthesis conclusions.
Coherence	Examines the extent to which findings are cohesive across different endpoints that are known/expected to be related to, or dependent on, one another (e.g., based on known biology of the organ system or disease, or mechanistic understanding such as toxicokinetic/dynamic understanding of the chemical or related chemicals). In some instances, additional analyses of mechanistic evidence from research on the chemical under review or related chemicals that evaluate linkages between endpoints or organ-specific effects may be needed to interpret the evidence. These analyses may require additional literature search strategies. Syntheses will consider potentially related findings, both within and across studies, particularly when relationships are observed within a cohort or within a narrowly defined category (e.g., occupation, strain or sex, life stage of exposure). Syntheses will emphasize evidence indicative of a progression of effects, such as temporal- or dose-dependent increases in the severity of the type of endpoint observed.
Natural experiments	Specific to epidemiology studies and rarely available, this examines effects in populations that have experienced well-described, pronounced changes in exposure to the chemical of interest (e.g., blood lead levels before and after banning lead in gasoline).

In addition, to the extent the data allowed, the syntheses discuss analyses relating to potential susceptible populations,[5] based on knowledge about the health outcome or organ system affected, demographics, genetic variability, life stage, health status, behaviors or practices, social determinants, and exposure to other pollutants (see Table 3-5). Consideration of susceptible life stages and populations are considered as previously described U.S. EPA (2014); (U.S. EPA, 2015).

[5]Various terms have been used to characterize populations that may be at increased risk of developing health effects from exposure to environmental chemicals, including "susceptible," "vulnerable," and "sensitive." Further, these terms have been inconsistently defined across the scientific literature. The term susceptibility is used in this protocol to describe populations at increased risk, focusing on biological (intrinsic) factors, as well as social and behavioral determinants that can modify the effect of a specific exposure. However, certain factors resulting in higher exposures to specific groups (e.g., proximity, occupation, housing) may not be analyzed to describe potential susceptibility among specific populations or subgroups.

Briefly, a targeted literature search was conducted using the overall arsenic literature database and modifying factors were evaluated.

Table 3-6. Individual and social factors that may increase susceptibility to exposure related health effects

Factor	Examples
Demographic	Gender, age, race/ethnicity, education, income, occupation, geography
Genetic variability	Polymorphisms in genes regulating cell cycle, DNA repair, cell division, cell signaling, cell structure, gene expression, apoptosis, and metabolism
Life stage	In utero, childhood, puberty, pregnancy, women of childbearing age, old age
Health status	Pre-existing conditions or disease such as psychosocial stress, body mass index, frailty, nutritional status, chronic disease
Behaviors or practices	Diet, mouthing, smoking, alcohol consumption, pica, subsistence or recreational hunting and fishing
Social determinants	Income, socioeconomic status, neighborhood factors, health care access, and social, economic, and political inequality
Women and men of reproductive age	Preconception and early fetal development (e.g., females who are in the early pregnancy but are not yet aware of their pregnancy)

Evidence syntheses are based on studies of *high* and *medium* confidence. As previously described, these syntheses articulate the strengths and the weaknesses of the available evidence organized around the considerations described in Table 3-4 as well as issues that stem from the evaluation of individual studies (e.g., concerns about bias or sensitivity). If possible, results across studies are compared using graphs and charts or other data visualization strategies. The analysis typically includes examination of results stratified by any or all the following: study confidence classification (or specific issues within confidence evaluation domains), population, exposures (e.g., level, patterns [intermittent or continuous], duration, intensity), sensitivity (e.g., low vs. high), and other factors that were identified in the refined evaluation plan (e.g., sex, life stage, or other demographics). Study sensitivity assesses whether factors in the study's design and conduct may reduce its ability to observe an effect, if present. The number of studies and the differences encompassed by the studies determines the extent to which specific types of factors can be examined to stratify study results.

Evidence integration conclusions are summarized in an evidence profile table for each hazard using the considerations outlined in Table 3-6. This process is similar to that used by GRADE (Morgan et al., 2016); (Guyatt et al., 2011); (Schünemann et al., 2011), which arrives at an overall level of confidence conclusion based on considering the body of evidence. The evidence profile table summarizes the judgments and their evidence basis. Judgments are reached after group discussion by the assessment team and independent review by the systematic review experts within EPA.

The analyses of each consideration in Table 3-6 are used to develop a strength-of-evidence judgment. Table 3-7 provides the judgments for each category and the criteria that guide how to apply the judgments. Briefly, the terms *robust* and *moderate* are standardized characterizations for judgments on the extent of support provided by human studies that the health effect(s) results from chemical exposure. These terms are differentiated by the quantity and quality of information available to rule out alternative explanations for the results. The term *slight* indicates situations in which there is some evidence indicating an association within the evidence stream, but substantial uncertainties in the data prevent stronger judgments from being drawn. *Indeterminate* reflects evidence-stream judgments when no studies are available, or situations in which the evidence is inconsistent and/or primarily of *low* confidence. *Compelling evidence of no effect* represents a situation in which extensive evidence across a range of populations and exposures has identified no effects/associations. This scenario is seldom used because it requires a high degree of confidence in the conduct of individual studies, including consideration of study sensitivity, and comprehensive assessments of health outcomes and life stages of exposure.

Table 3-7. Considerations that inform judgments regarding the strength of the human evidence

Consideration	Increased evidence strength	Decreased evidence strength
Evidence synthesis scenarios that do not warrant an increase or decrease in evidence strength will be considered "neutral" and do not need to be described in the evidence profile table.		
Risk of bias (across studies)	An evidence base of *high*- or *medium*-confidence studies increase strength.	An evidence base of mostly *low*-confidence studies decrease strength. Decisions to increase strength for other factors should generally not be made if there are serious concerns for risk of bias.
Study sensitivity (across studies)	An evidence base of studies with mostly *good* or *adequate* sensitivity increases strength.	An evidence base of studies with poor sensitivity typically decreases confidence in null conclusions. Conversely, an evidence base of studies with mostly poor sensitivity may increase evidence strength in cases where an association is identified because the most common predicted impact of study insensitivity is towards the null.
Consistency	The similarity of findings for a given outcome (e.g., of a similar magnitude, direction) across independent studies or experiments increases strength, particularly when consistency is observed across populations (e.g., location) or exposure scenarios in human studies.	Unexplained inconsistency (conflicting evidence) decreases strength. Strength should not be decreased if discrepant findings can be explained by study confidence conclusions; variation in population, sex, and life stage; exposure patterns (e.g., intermittent, or continuous); levels (low or high); duration; or intensity.
Strength (effect magnitude) and precision	Evidence of a large magnitude effect (considered either within or across studies), can increase strength. Precise results from individual studies or across the set of studies, noting that biological significance is prioritized over statistical significance.	The presence of small effects is not typically used to decrease confidence in a body of studies. However, if effect sizes that are small in magnitude are concluded not to be biologically significant, or if there are only a few studies with imprecise results, then strength is decreased.
Biological gradient/dose-response	Evidence of dose-response relationship, which may be demonstrated across studies or within studies.	A lack of dose-response relationship when expected based on biological understanding and having a wide range of doses/exposures evaluated in the evidence base can decrease strength. If the data are not adequate to evaluate a dose-response pattern, then strength is neither increased nor decreased.

Consideration	Increased evidence strength	Decreased evidence strength
Coherence	Biologically related findings within an organ system, or across populations (e.g., sex), particularly when a temporal- or dose-dependent progression of related effects is observed within or across studies.	An observed lack of expected coherent changes (e.g., well-established biological relationships), particularly when observed for multiple related endpoints, will typically decrease evidence strength. Decision to decrease depends on the strength of the expected relationship(s), and considers factors (e.g., dose and duration of exposure) across studies of related changes.

Table 3-8. Framework for evidence judgments from studies in humans

Within-stream strength-of-evidence judgment	Description
Robust (⊕⊕⊕) … evidence in human studies	A set of *high*- or *medium*-confidence independent studies reporting an association between the exposure and the health outcome, with reasonable confidence that alternative explanations, including chance, bias, and confounding, can be ruled out across studies. The set of studies is primarily consistent, with reasonable explanations when results differ; an exposure-response gradient is demonstrated; and the set of studies includes varied populations. Additional supporting evidence, such as associations with biologically related endpoints in human studies (coherence) or large estimates of risk, may increase confidence but are not required. In exceptional circumstances, a finding in one study may be considered *robust*, even when other studies are not available (e.g., analogous to the finding of angiosarcoma, an exceedingly rare liver cancer, in the vinyl chloride industry). Mechanistic evidence from exposed humans or human cells, if available, may add support informing considerations such as exposure response, temporality, coherence, and MOA, thus raising the level of certainty to *robust* for a set of studies that otherwise would be described as *moderate*.
Moderate (⊕⊕⊙) … evidence in human studies	A smaller number of studies (at least one *high*- or *medium*-confidence study with supporting evidence), or with some heterogeneous results, that do not reach the degree of confidence required for *robust*. For multiple studies, there is primarily consistent evidence of an association, but there may be lingering uncertainty due to potential chance, bias, or confounding. For a single study, there is a large magnitude effect or dose-response gradient observed in a study where exposure is well characterized. Supporting evidence could include associations with related endpoints, including mechanistic evidence from exposed humans or human cells, if available, based on considerations such as exposure response, temporality, coherence, and MOA, thus raising the level of certainty to *moderate* for a set of studies that otherwise would be described as *slight*.
Slight (⊕⊙⊙) … evidence in human studies	One or more studies reporting an association between exposure and the health outcome, where considerable uncertainty exists. In general, only *low*-confidence studies may be available, or considerable heterogeneity across studies may exist. Supporting coherent evidence is sparse. Strong biological support from mechanistic evidence in exposed humans or human cells may also be independently interpreted as *slight*. This category serves primarily to encourage additional study where evidence does exist that might provide some support for an association, but for which the evidence does not reach the degree of confidence required for *moderate*.
Indeterminate (⊙⊙⊙) … evidence in human studies	No studies available in humans or situations when the evidence is inconsistent and/or primarily of *low* confidence.
Compelling evidence of no effect (- - -) … in human studies	Several *high*-confidence studies showing null results (for example, an odds ratio of 1.0), ruling out alternative explanations including chance, bias, and confounding with reasonable confidence. Each of the studies should have used an optimal outcome and exposure assessment and adequate sample size (specifically for higher exposure groups and for susceptible populations). The set should include the full range of levels of exposures that human beings are known to encounter, an evaluation of an exposure-response gradient, and an examination of at-risk populations and life stages.

Based on the totality of the evidence, this stage culminates in a narrative that summarizes the conclusions regarding each potential health effect (i.e., each noncancer health effect and specific

type of cancer, or broader grouping of related outcomes as defined in the evaluation plan). The evidence narrative includes:

- A descriptive summary of the primary conclusions about the potential for health effects in exposed humans;
- A summary of key evidence supporting these conclusions, highlighting the primary drivers of these judgments and any notable issues (e.g., data quality; coherence of the results), and a narrative expression of confidence across these conclusions;
- Information on the conditions of expression of these health effects (e.g., exposure routes, levels of exposure, etc.);
- Indications of potentially susceptible populations or life stages;
- A summary of key assumptions used in the analysis, which are often based on EPA guidelines; and
- Strengths and limitations of the conclusions, including key uncertainties and data gaps.

For evaluations of carcinogenicity consistent with EPA's Cancer Guidelines (U.S. EPA, 2005a), one of EPA's standardized cancer descriptors is used as a shorthand characterization of the evidence integration narrative, describing the overall potential for carcinogenicity. These are (1) *carcinogenic to humans*, (2) *likely to be carcinogenic to humans*, (3) *suggestive evidence of carcinogenic potential*, (4) *inadequate information to assess carcinogenic potential*, or (5) *not likely to be carcinogenic to humans*. Because bladder cancer and lung cancer are accepted hazards, the corresponding cancer descriptors for these health outcomes are carcinogenic to humans.

Currently, EPA does not have guidance on the use of standardized descriptors for noncancer hazards, so none will be applied, although conclusions indicated confidence in the body of evidence (e.g., "evidence demonstrates," "evidence suggests," or through use of symbols ⊕⊕⊕, ⊕⊕⊙, ⊕⊙⊙, or ⊙⊙⊙) with exposure context provided.

4. PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL DESCRIPTIVE SUMMARY AND EVALUATION

Physiologically based pharmacokinetic (PBPK) models for inorganic arsenic are important for describing exposure-internal dose relationships and, thus, informing dose-response estimates. Judgments on the suitability of a model are separated into two categories: scientific and technical. The scientific criteria focus on whether the biology, chemistry, and other information available for chemical mode(s) of action (MOA[s]) are justified (i.e., preferably with citations to support use) and represented by the model structure and equations. The scientific criteria are judged based on information presented in the publication or report that describes the model and do not require evaluation of the computer code. Preliminary technical criteria include availability of the computer code and completeness of parameter listing and documentation. Studies that meet the preliminary scientific and technical criteria are then subjected to an in-depth technical evaluation, which includes a thorough review and testing of the computational code. The in-depth technical and scientific analyses focus on the accurate implementation of the conceptual model in the computational code, use of scientifically supported and biologically consistent parameters in the model, and reproducibility of model results reported in journal publications and other documents. This approach stresses (1) clarity in the documentation of model purpose, structure, and biological characterization; (2) validation of mathematical descriptions, parameter values, and computer implementation; and (3) evaluation of each plausible dose metric. The in-depth analysis is used to evaluate the potential value and cost of developing a new model or substantially revising an existing one as a component of the draft assessment or by publication in a peer-reviewed journal.

The development of useful biologically based dose-response models has proved challenging because inorganic arsenic mediates its toxicity through a range of metabolites, and their roles about specific adverse effects are not clear (Clewell et al., 2007). PBPK models have been developed specifically for inorganic arsenic exposure (Mann et al., 1996a); (Mann et al., 1996b); (Yu, 1999b); (Gentry et al., 2004); (El-Masri and Kenyon, 2008). (Mann et al., 1996a) provided a PBPK model for hamsters and rabbits, and (Mann et al., 1996b) described an extension of this model for humans, but model code was not available for the human version. For the (Yu, 1999a) human model, it is not clear how model optimization was performed. The (Gentry et al., 2004) mouse model used the same partition coefficients used by (Mann et al., 1996a). (Liao et al., 2008) described the combination of a PBPK model for human children with a Weibull dose-response model. These models were evaluated using the approach described above (see Appendix A), and the (El-Masri and Kenyon, 2008) model was chosen as the most appropriate because it is peer reviewed,

optimized, and specific to humans. This PBPK model will be used to obtain a common exposure metric for use in dose-response meta-analyses.

The El-Masri and Kenyon PBPK model (El-Masri and Kenyon, 2008) for arsenic was developed for a human male and incorporates the different forms of arsenic. These forms include arsenate (As[V]), arsenite (As[III]), monomethylarsenic acid (MMA[V]), dimethylarsenic acid (DMA[V]), monomethylarsonous acid (MMA[III]), and dimethylarsinous acid (DMA[III]) (El-Masri and Kenyon, 2008). There are no available models for women of reproductive age, pregnant women, or children. This model has eight compartments: lung, liver, gastrointestinal (GI) tract (lumen and tissue), kidney, muscle, brain, skin, and heart (see Figure 4-1). The physiological parameters came from the literature (Brown et al., 1997). The arsenic species and their metabolites are distributed in the systemic circulation simultaneously. Metabolism of inorganic arsenic, and its metabolites was described in the PBPK model as a series of reduction and oxidative methylation steps. Parameters for the metabolic rate equations were estimated using published literature values, or via optimization of simulations to data. The routes of absorption are oral, dermal, and inhalation. Dermal absorption and urinary elimination are described in the model as first-order processes. The partition coefficient came from the literature (Benramdane et al., 1999); (Saady et al., 1989) and is a single coefficient for total As. The authors then made a correction using the ratio of the specific distribution of As species and the total measured as reported by (Saady et al., 1989). The authors conclude that the partition coefficients were relatively similar to those used by (Yu, 1999b). The authors assumed flow-limited diffusion in each compartment and distinguished the ionization levels of MMA and DMA.

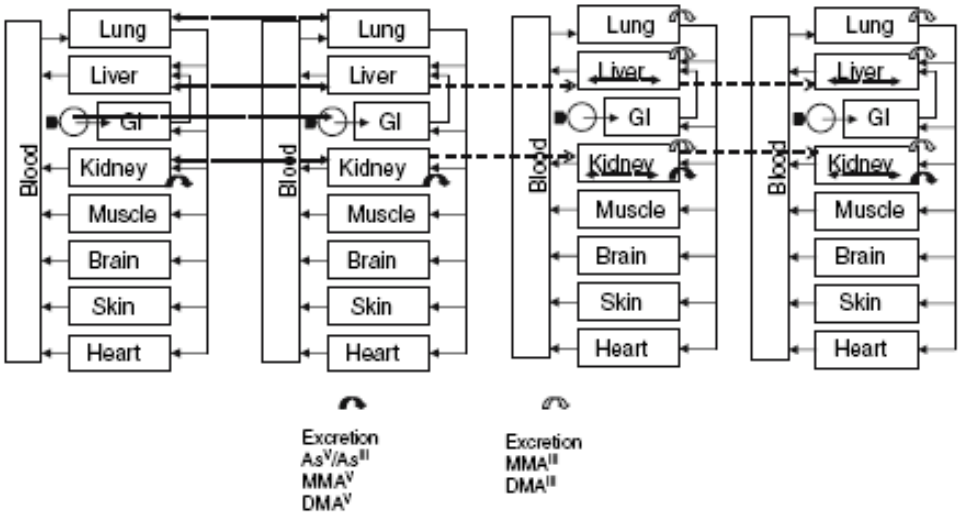


Figure 4-1. Conceptual representation of physiologically based pharmacokinetic (PBPK) model.

Source: (El-Masri and Kenyon, 2008).

For the metabolites, the authors suggested inhibitory effects of As(III) on the methylation of MMA(III) to DMA(V), MMA(III) on the methylation of As(III) and the methylation of MMA(III) to DMA(V), and MMA(III) on the methylation of As(III) to MMA, which were modeled as noncompetitive inhibition (see Figure 4-2).

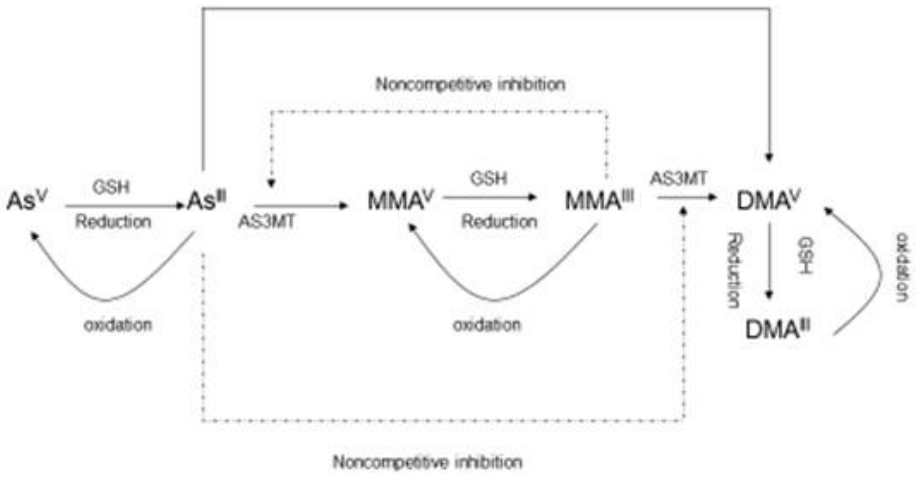


Figure 4-2. Metabolism pathways described in the literature.

Source: (El-Masri and Kenyon, 2008).

The (El-Masri and Kenyon, 2008) model was run using the Simulink platform, with parameter optimization conducted using MATLAB® against two large data sets (~11,000 and 500 subjects in Bangladesh and Nevada, respectively; see (Buchet et al., 1981b), (Buchet et al., 1981a), and (Lee et al., 1999), which provided matched individual chronic inorganic arsenic drinking water exposure and urinary excretion data. Overall, the evaluation of the model showed a better prediction at a low dose than at a high dose (El-Masri et al., 2018b). Results illustrated the PBPK model's use in evaluating the contribution of arsenic in food and water to total exposure and demonstrated the model's value in reconstructing human exposures to inorganic arsenic, particularly in individuals exposed to relatively low levels of arsenic in water or food, see Figure 4-3; (El-Masri et al., 2018b).

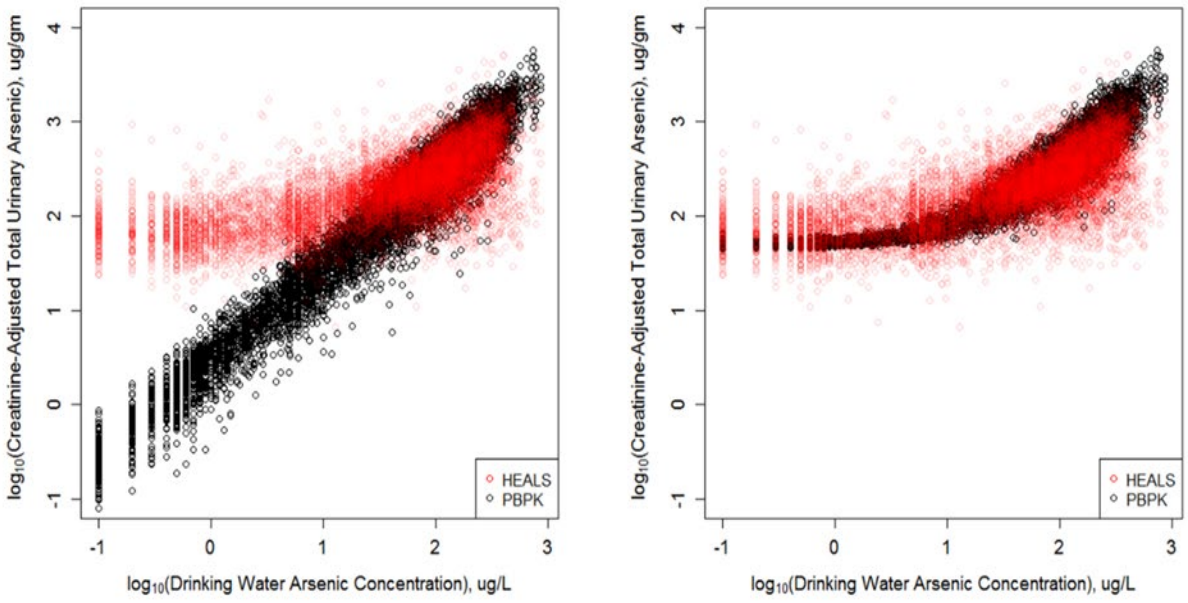


Figure 4-3. Relationship between arsenic water levels and physiologically based pharmacokinetic (PBPK) model-predicted creatinine-adjusted total urinary arsenic concentrations for the Health Effects of Arsenic Longitudinal Study (HEALS) data set. Left: well water as the only arsenic intake source. Right: combined well-water and dietary exposure as the arsenic intake source.

5. DOSE-RESPONSE ASSESSMENT: SCREENING EPIDEMIOLOGICAL DATA SETS, ESTIMATING INTAKE DOSES, MODELING DOSE-RESPONSE DATA, AND DERIVING REFERENCE VALUES

Selection of specific data sets for dose-response assessment and performance of the dose-response assessment is conducted after hazard identification is complete and involves database- and chemical-specific biological judgments that are beyond the scope of this protocol. But they are discussed in existing EPA guidance and support documents, especially EPA's *Benchmark Dose Technical Guidance* (U.S. EPA, 2012), EPA's *Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002b), *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), and *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA, 2005b). This section of the protocol provides an overview of the process EPA will use when conducting the inorganic arsenic (iAs) dose-response assessment and deriving toxicity values (e.g., slope factors and RfDs), including further identifying health outcomes that can support dose-response modeling, the estimation of intake doses and adjusted cases from study data, and preferred approaches to modeling the adjusted dose-response data.

5.1. INITIAL SCREENING ANALYSES

Given the extensive epidemiological evidence base of iAs studies, EPA developed and applied a preliminary approach for data selection and modeling to help identify the endpoints and studies that warrant more complex, rigorous dose-response analyses (Hobbie et al., 2020). Studies for health outcomes with *Robust* and *Moderate* evidence were first reviewed for their suitability for dose-response modeling based on key considerations that are summarized in Table 5-1.

Table 5-1. Rating criteria for inorganic arsenic exposure or dose response data sets for prioritizing studies for dose response analysis

Consideration	Criteria
Health Outcome	The focus is on health outcomes identified as having Robust or Moderate evidence using criteria described in Section 3.11. While mortality studies were not excluded, incidence data is preferred.
Exposure ascertainment method	Individual measurement or small group averages are preferred over just location of residence/exposure or large group averages.
Exposure reporting	Summary statistics such as averages and measures of dispersion/variance are preferred over just ranges. Ranges are preferred over absolute values without variance information.[a]
Estimates control for smoking, gender, age, and other key covariates	Adjusted estimates that include important covariates are preferred over unadjusted estimates. Smoking status is a critical covariate that requires adjustment.
Number of exposure groups	Studies using more exposure groups are preferred, particularly over studies for which the limited number of exposure groups precludes adequate exposure-response modeling.
Number of subjects (referents) and cases reported	Explicit reporting of the number of subjects and cases is preferred over just statistical summaries (RR, SMRs, etc.,).
Exposure/biomarker metric	Subject-specific cumulative intake and creatinine corrected urinary intake biomarker[b] data are preferred over cumulative exposure, group exposure or historical exposure measurements.
Exposure timing and duration	Explicit ascertainment of exposure histories (timing, duration) preferred over studies that do not ascertain or reported exposure history (e.g., studies that report exposure levels for just one time point).
Representativeness of referent group/controls	Well documented reports that compare referent to exposed groups for key variables preferred over reports that do not provide such documentation or document major differences between referent and exposed groups.
Sufficient number of subjects, cases	A sufficient number of cases to conduct reliable statistical analyses (most applicable to cohort cancer studies) preferred; desirable to have >~ 5 cases/exposure group.

[a]Studies that report "0" for control exposures were excluded from consideration for RRB-SP derivations due to lack of a valid referent group background exposure estimate to use for the denominator of the RRB-SP equation.
[b]An exception is when the subject health outcome studied is associated with renal impairment that could substantially impact clearance rates resulting in higher blood creatinine but lower urinary creatinine in cases relative to controls.

After applying these Table 5-1 criteria, a large number of data sets that varied considerably in their suitability for dose-response analysis remained. Evaluating this large number of datasets using the methods described in Section 5.2 was problematic given the impracticality of converting all exposure metrics reported in the studies to a single intake dose metric and performing complex statistical dose-response modeling for all outcomes. To provide additional context for prioritized health outcomes, studies, and data sets, a preliminary analytical approach was developed that utilizes the exposure metrics and relative risk estimates reported by the study authors (Hobbie et al., 2020). Benchmark dose modeling is used to obtain a study-specific estimate of the exposure

level associated with a given relative risk (RRE) over the risk estimated at a US background level (RRE-US) and over the risk estimated at the study's reference group exposure level (RRE-SP). These RRE-US and RRB-SP estimates are divided by an estimated US or study population background exposure level (in terms of the study-specific exposure metrics) to obtain what will be referred to as RRB-US (relative risk exposure vs. US background exposure) and RRB-SP (relative risk exposure vs. the study population's background exposure) ratio; the lower a RRB value, the greater the concern. The RRB-US estimates are the focus here as they are more relevant for low exposure populations like the US. However, it is useful to compare them to RRB-SP estimates, which involve less extrapolation and, consequently, less model uncertainty and less RRB estimation variability. In general, RRB-SP estimates from iAs epidemiological studies are consistent with the RRB-US estimates with respect to what they suggest about potential differences in arsenic's relative potencies across health outcomes (Hobbie et al., 2020).

For the purposes of this analysis, the data sets identified after applying the Table 5-1 rating criteria were subjected to additional considerations that included:

- the type of response data reported (published relative risk [RR] estimates are necessary for the RRB analysis),
- the exposure or biomarker metric used in the study (e.g., drinking water vs. urine, historic exposure vs. cumulative exposure [preferred]), and
- whether the study provided the necessary quantitative data for modeling (e.g., number of cases and controls for all exposure groups).

In total, more than 250 separate data sets were identified as suitable for the purposes of the screening analysis and were modeled using EPA's Benchmark Dose Software (www.epa.gov/bmds) (Hobbie et al., 2020). Points of departure were based on the maximum likelihood estimate of the exposure or biomarker metric reported in a study that would increase the relative risk by 20% [$RRE\text{-}US_{22}$ or $RRE\text{-}SP_{20}$][6] to derive RRB-US and RRB-SP values for each data set. The U.S background exposure estimates used to derive the $RRE\text{-}US_{20}$ values are shown in Table 5-2.[7]

[6]The RRE_{20} is not meant to represent a "clinically significant" endpoint or to have any other policy-relevant interpretation other than for purposes such as those described for this RRB analysis, particularly the identification of studies and health outcomes that warrant further consideration for additional dose-response analysis. The 20% effect level was chosen for this comparative analysis after examination of the effect sizes and exposure ranges of the input data sets. A key consideration was that EPA wanted the output RRE values to be in or near the range of the input data as frequently as possible; that is, extrapolating far outside the range of data was to be avoided.

[7]This results in a unitless RRE_{20}/U.S. background ratio that can be compared across studies regardless of the exposure metric reported in the study.

Table 5-2. United States estimates for different arsenic exposure and dose metrics

Exposure metric	Units	U.S. central tendency	U.S. high	Basis for U.S. estimate
Drinking water concentration	µg/L	1.5	15.4	Median, 95th percentile county mean As in drinking water (USGS, 2011)
Cumulative exposure from drinking water	µg/yr-L	75	770	1.5 µg/L or 15.4 µg/L (see above) × 50 yrs
Daily intake	µg/d (water)	1.5	15.4	1.5 or 15 µg/L (see above) × 1.0 L/d (U.S. EPA, 2011)
Dietary intake	µg/d (food)	3.5	13.3	0.05 µg/kg-d mean or 0.19 µg/kg-d 95th percentile adult intake (Xue et al., 2010) 70-kg adult
	µg/d (food + water)	5	28.7	Sum of food and water intake
Cumulative intake	mg (cumulative intake, water)	27.4	281	1.5 µg/d or 15.4 µg/day (see above) × 50 yrs
	mg (cumulative intake, food + water)	91.3	524	5 µg/d or 28.7 µg/d (see above) × 50 yrs
Urine concentration (cr. Adj.)	µg As excretion/g creatinine	7.4	18.4	NHANES (2013–2014) median, 95th percentile (CDC, 2016)
Urine concentration	µg AS excretion/L urine	5	16.8	NHANES (2013–2014) median, 95th percentile (CDC, 2016)
Air	µg/m^3	0.00075	0.00156	https://cfpub.epa.gov/roe/indicator.cfm?i=90#8; EPA's ambient monitoring archive, arsenic data averaged between 2010 and 2013
Cumulative air	µg/m^3-yrs	0.0375	0.078	0.00075 µg /m^3 or 0.00156 µg /m^3 (above) × 50 yrs

To assist in prioritizing health outcomes for more in-depth dose-response analyses, EPA considered the number of suitable data sets available and estimated the average and median RRBs for each health outcome. Background lifetime risk was also a consideration because a 20% increase in the relative risk of a health outcome with a high background lifetime risk could have a more serious public health implication relative to a health outcome with a low background lifetime risk.

Figure 5-1 presents the results of the RRB screening analysis for RRB-US estimates. Immune effects and developmental neurocognitive effects were not included in the RRB analysis because of the absence of suitable studies reporting relative risk (RR) estimates[8] necessary for deriving RRB values that can be compared across studies.

[8]Principally due to an inappropriate study design (e.g., ecological epidemiology) or a lack of data needed for dose-response modeling (e.g., only regression coefficients provided, cases or number of subjects not reported, etc.).

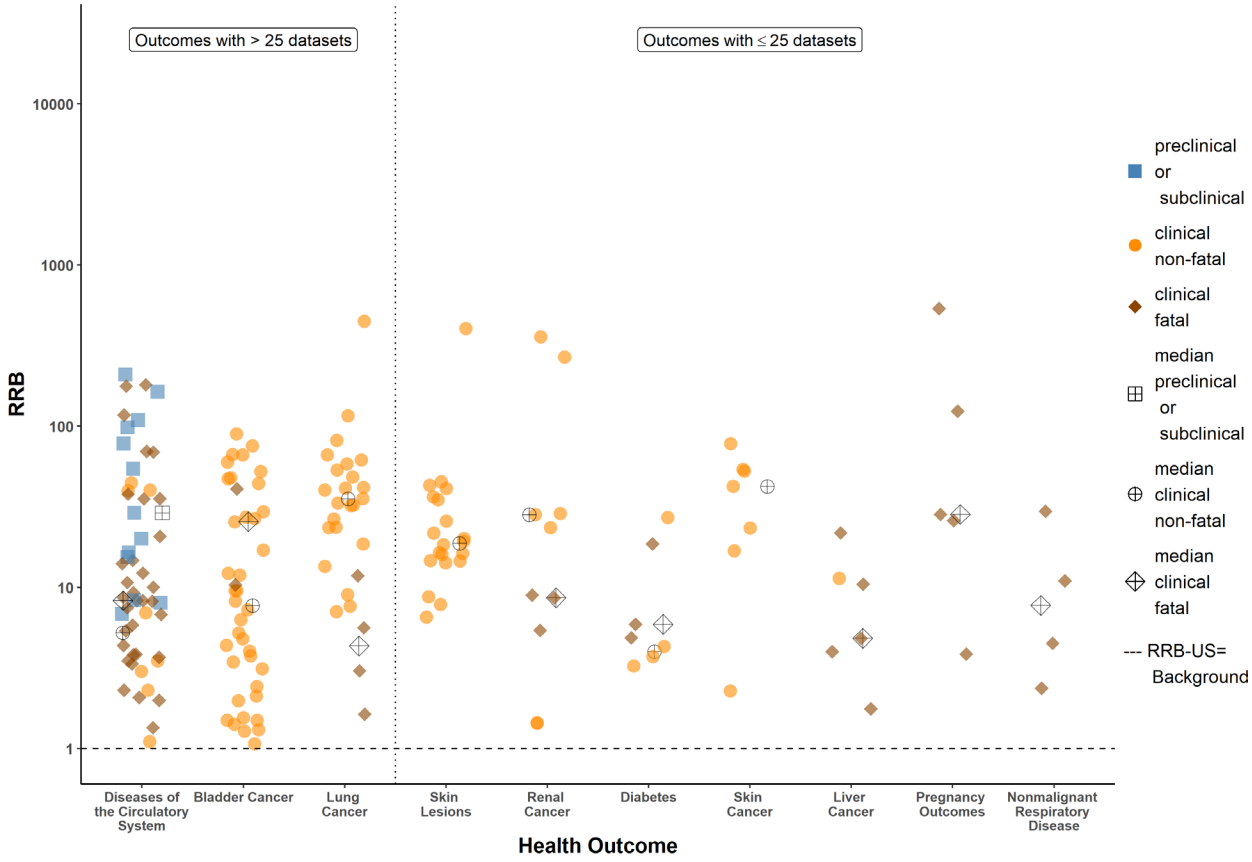


Figure 5-1. Individual Dataset (solid symbols) and median (crosshatch symbols) RRB-US estimates for health outcomes with >25 and ≤25 datasets supporting the derivation of RRE-US$_{20}$ estimates from studies for which the estimated RRE-US$_{20}$ estimate was not more than a factor of three below the central estimate for the lowest dose group or above the central estimate for the highest dose group of the study (Hobbie et al., 2020).

The results of this RRB analysis, along with the considerations described above, were then used to inform the selection of studies and data sets for further dose-response analysis (see Section 5.5). With respect to exposure context, out of the 12 health outcomes considered in the RRB analysis, diseases of the circulatory system, bladder cancer, and lung cancer had multiple individual study RRB values close to 1, with median RRB values near or below 10, indicating that exposures resulting in a 20% increase in relative risk were very close to U.S. background exposure levels for inorganic arsenic. These outcomes also had the largest databases suitable for further dose-response modeling (>25 data sets). Based on these RRB screening results, bladder cancer, lung cancer, disorders of the cardiovascular system, pregnancy and birth outcomes, and diabetes have been prioritized for additional dose-response analysis. Additionally, developmental neurotoxicity (i.e., developmental neurocognitive effects) have been identified as being particularly important to EPA Program Offices for benefit-cost analysis. Therefore, while the lack of published relative risk estimates necessary for the derivation of RRE$_{20}$ estimates did not allow for the

inclusion of the developmental neurotoxicity (i.e., developmental neurocognitive effects) in the RRB comparative analysis, a thorough dose-response analyses on key continuous variables associated with this health outcome are planned. Table 5-3 summarizes the status of each of the NRC hierarchy of outcomes proposed for inclusion in the assessment. Additional dose-response analyses, including analyses of potentially sensitive subgroups, will be performed on these health outcomes as appropriate given their respective databases (see Section 5.5).

Table 5-3. Status of (NRC, 2013) prioritization of health outcomes of concern for dose-response analysis in EPA's inorganic arsenic assessment

Health outcome	NRC tier	EPA SR	Strength-of-evidence	Proposed status for dose-response analysis in assessment
Lung cancer	1	No	Robust	✓
Bladder cancer	1	No	Robust	✓
Skin cancer	1	No	Robust	Not prioritized for dose-response based on RRB analysis
IHD and CVD	1	Yes	Robust	✓ U.S. lifetime extra risks estimated for fatal IHD, fatal CVD, and incidence of CVD (IHD + stroke).
Skin lesions	1	No	Robust	Not prioritized for dose-response based on RRB analysis
Prostate cancer	2	Yes	Slight	Not prioritized for dose-response based on hazard characterization
Renal cancer	2	Yes	Moderate	Not prioritized for dose-response based on hazard characterization
Diabetes	2	Yes	Robust	✓
Nonmalignant respiratory disease	2	Yes	Moderate	Not prioritized for dose-response based on hazard characterization
Pregnancy outcomes (infant morbidity)	2	Yes	Moderate	✓
Neurodevelopmental toxicity	2	Yes	Moderate	✓ Small database, included for use in benefit-cost analyses by other Program Offices
Immune effects	2	Yes	Moderate	Not prioritized for dose-response (no suitable data sets for analysis).
Pregnancy outcomes (fetal loss, stillbirth, and neonatal mortality)	3	Yes	Moderate	✓
Liver cancer	3	Yes	Moderate	Not prioritized for dose-response based on hazard characterization
Pancreatic cancer	3	Yes	Slight	Not prioritized for dose-response based on hazard characterization
Renal disease	3	Yes	Slight	Not prioritized for dose-response based on hazard characterization
Hypertension	3	Yes	Robust	Not prioritized as a separate DCS outcome based on existence of a larger database and stronger dose-response for other DCS outcomes, IHD, and CVD.
Stroke	3	Yes	Robust	✓ U.S. lifetime extra risks estimated for CVD (IHD + stroke) incidence and fatality

SR= systematic review; CVD = cardiovascular disease; IHD = ischemic heart disease; DCS = diseases of the circulatory system.

5.2. DOSE-RESPONSE OVERVIEW

For the quantification of noncancer values, this assessment will derive oral reference dose (RfD) estimates, which are "estimates, with uncertainty spanning perhaps an order of magnitude, of an exposure to the human population (including susceptible subgroups) that is likely to be without an appreciable risk of deleterious health effects over a lifetime" (U.S. EPA, 2002b). Inhalation reference concentration (RfC) estimates will not be derived in this assessment.

RfD values will preferably be derived using the Bayesian meta-regression methods (Section 5.2.1), but if infeasible due to data quality or poor model fits, a traditional BMD approach (i.e., selecting a single best model from individual dose-response datasets) will be used. In addition to RfD values, estimations of risk will be given at various dose levels. For the combination of studies in a meta-analysis and for the consideration of background intake, the exposure information generally reported in the epidemiologic literature (e.g., drinking water concentrations) will be converted to μg/kg-day intake doses (see *Estimating a common intake dose from study data*). In deriving these intake dose estimates, EPA will consider all available and relevant study/population-specific routes of exposures, as well as the pertinent variabilities and uncertainties in factors that impact intake (dose) extrapolations and relative risk estimations (see *Variability and uncertainty in dose-response analyses*).

For priority cancer health outcomes, EPA will derive, upper-bound U.S. population-specific risk estimates with confidence intervals (that account for identified sources of variability and uncertainty to the extent the data can support such analyses) from epidemiological data over a broad range of inorganic arsenic intake doses (μg/kg-day) above U.S. background levels. If the dose-response relationships are deemed sufficiently linear to background levels of exposure in the U.S., those linear relationships will be provided so that approximations of the mean and upper-bound risks for cancer health outcomes can be derived. In cases of non-linear dose-response relationships, flexible polynomial approximations will be provided. The upper-bound linear relationships will be analogous to oral slope factor (OSF) estimates that EPA has historically provided for unit cancer risks. The OSF represents the upper-bound lifetime risk from chronic ingestion of a chemical per unit of mass consumed per unit body weight per day (expressed as $[\mu g/kg\text{-}day]^{-1}$).

Exposure and use of biomarkers

A careful assessment of the ability of reported exposure and intake measures to characterize exposure status, exposure levels, and dose estimates accurately and correctly is essential for the dose-response analysis of epidemiological studies (U.S. EPA, 2022). EPA's preferred dose metric for the assessment of epidemiological studies is daily or cumulative dose of iAs for individual subjects, with information related to the timing and intensity of exposure. Most epidemiological studies, however, simply provide group summary estimates (e.g., ranges or means and standard deviations) of exposures (e.g., μg/L in drinking water, cumulative μg-year/L) or

intake (e.g., µg/kg-day or cumulative mg intake). When individual data for exposures or intakes associated with responses are not available, EPA prefers studies that (1) report estimates of exposure or intake for multiple small groups and (2) characterize exposure or intake estimates in terms of means or medians with an indication of variation or dispersion (e.g., standard deviations or quartiles). When only exposure ranges are provided, group mean exposure levels will be estimated, assuming a single lognormal distribution or a mixture of multiple distributions and using whatever information is available to make informed judgments regarding the minimum and maximum exposure levels if they are not reported. Studies that provide relevant estimates of consumption rates (e.g., liters of water consumed per day) and exposure duration (e.g., years of consumption from water source) for the study population are preferred for accurate characterization of the dose-response relationship.

For the screening level of modeling described in Section 5.1, EPA relied on the intake or exposure metrics reported by the authors. Consistent with (NRC, 2013) and (NASEM, 2019) recommendations to account for total inorganic arsenic dose and to allow more studies to be included in meta-analyses, EPA will derive a common dose metric for use in multiple study meta-regression analyses described in this section. To do so, EPA will use data from a range of sources to (1) calculate daily lifetime arsenic intake from water (where water is the exposure medium) and (2) add a contribution from dietary exposures (see *Estimating a common intake dose from study data*).

Consistent with (NRC, 2013) recommendations, EPA focused its dose-response analyses on epidemiological studies that include observations associated with exposures ranging from US background concentrations to 100 µg/L in drinking water, which encompasses exposures commonly found in the United States. Data sets comprising substantial proportions of subjects with low-to-moderate exposures and more highly exposed groups were also evaluated, however.

Another important aspect of exposure uncertainty is related to how well the reported exposure measurements represent the etiologically relevant time window. EPA prefers studies that document the exposure histories for study subjects and provide estimates of cumulative exposure during a relevant life stage or for the entire lifespan until diagnosis. Such studies are important to this assessment because risks associated with iAs are influenced by early life-stage exposure, including in utero exposure (Steinmaus et al., 2013).

Evaluating biomarkers with a PBPK model

PBPK models are helpful for associating doses and exposure levels with biomarkers of body burden commonly reported in iAs studies, particularly urinary arsenic levels. As discussed in Section 4, EPA has developed and validated PBPK model for iAs (El-Masri and Kenyon, 2008); (El-Masri et al., 2018b); (El-Masri et al., 2018a) using urinary arsenic data from two large populations, one U.S. and one Bangladeshi, chronically exposed to arsenic. EPA will use the model in this assessment to:

- interpret the results of biomonitoring data (e.g., amount of arsenic excreted in urine) to better inform estimates of external exposures and daily intake, through back-estimation of levels of exposure and dose consistent with measured biomarkers levels; and

- normalize the data reported in multiple studies to a common metric of exposure or dose for the purposes of a meta-regression analysis.

Characterizing dose–response relationships down to the background concentrations

In their recommendations regarding EPA's iAs assessment, the NRC (2013) stated that it "…assumes that the needs of assessing health risks can be facilitated by characterizing dose–response relationships down to the background concentrations by using observed data." For the purposes of this assessment, the U.S. "background dose" is defined as the sum of the median U.S. dietary and median U.S. water consumption doses. This U.S. background dose is presumed to be associated with "background" risk levels reported for various health outcomes in the U.S. In this assessment, EPA will focus on deriving estimates of extra risk above an estimate of the risk at zero dose, assuming that a 0.0365 µg/kg-day background iAs intake (dose), 0.02 µg/kg-day from diet (Xue et al., 2010) and 0.0165 µg/kg-day from drinking water,[9] is associated with background levels of risk in the U.S. population. The (NASEM, 2019) supported this approach. The (NRC, 2013) committee "…judged that urinary arsenic concentrations of 1–5 µg/L (summing inorganic, monomethyl, and dimethyl arsenic forms) is a reasonable estimate [of background concentrations] for the US population."[10] EPA's PBPK model indicates that this urinary range of total arsenic is consistent with the 0.0365 µg/kg-day US background intake (dose) of inorganic arsenic, that is assumed for the purposes of the dose-response analyses conducted in this assessment.

Estimating a common intake dose from study data

Study exposure information will be converted to a common intake dose metric (µg/kg-day) for use in dose-response analysis and for the purposes of a multiple study meta-regression analysis. Arsenic intake from food and water will be summed to provide estimates of total arsenic daily dose

[9]Median U.S. County average inorganic arsenic drinking water concentration (1.5 µg/L) from USGS data (Mendez et al., 2017) multiplied by the average water intake in the U.S. population of 0.011 L/kg-day (U.S. EPA, 2019), Table 3-1, "All Ages" gives an estimated intake of iAs from water of 0.0165 µg/kg-day. Background intake from air exposure, estimated to be less than 9 x 10-3 µg/kg/day, does not have a marked impact on extra risk calculations (Allen et al., 2020b) and is therefore set to 0 µg/kg/day for the purposes of this assessment.

[10]This background level was estimated from published epidemiological studies (Karagas et al., 2001); (Navas-Acien et al., 2009);(Zheng et al., 2013) and values obtained from the National Health and Nutrition Examination Survey (NHANES) that exclude populations that consume substantial amounts of fish (to eliminate a large contribution by arsenobetaine and other seafood arsenicals in the NHANES total urinary arsenic measurements) (Navas-Acien et al., 2011). (NRC, 2013) stated that background concentrations are assumed to derive from a "variety of sources that potentially include very low concentrations in water, diet, dust, and other sources."

over a lifetime.[11] The procedures and formulas for doing this vary with the multiple types of exposure metrics reported in studies. For example, when responses are reported in relation to well water concentrations the following formula applies.

$$dose = DI + f \times (WCR \times WE) + (1 - f) \times (WCR \times LE) \quad (1)$$

DI = dietary intake (average daily μg/kg);

f = fraction of time (over lifetime up through the study) spent consuming well water (unitless);

WCR = water consumption rate (L/kg);

WE = well water concentration (μg/L); and

LE = low-end water concentration (μg/L).

Values used would ideally come from study-specific data but could also be drawn from other suitable population-specific sources (e.g., exposure factors handbook). Monte Carlo analysis will be performed on assumed/estimated distributions for all exposure factors to characterize uncertainty.

Variability and uncertainty in dose-response analyses

Assessing variability and uncertainty is important in characterizing risk. Variability represents the diversity or heterogeneity of a factor that can influence the response within an individual or across a population. Uncertainty represents unavailable or incomplete information on a specific variable that can influence the analyses. Regarding variability, many factors are instrumental in determining an individual's risk from exposure, including concurrent background exposures to other chemicals and the individual's biological susceptibility due to genetic, lifestyle, health, and other factors (U.S. EPA, 1992). In turn, population responses to chemical exposures depend on the distribution of these varying individual determinants in the population, including by life stage. The *IRIS Toxicological Review of Inorganic Arsenic* relies on observational epidemiological data for the dose-response analysis; Section 3.3 of the iAs assessment provides more details on how variability and uncertainty were considered and addressed in the dose-response analyses for inorganic arsenic.

To convert study-specific exposure metrics to intake dose estimates used in the dose-response analyses (see *Estimating a common intake dose from study data*), each study was

[11]For the meta-analyses, background inhalation exposure to arsenic is assumed zero as it is negligible relative to the drinking water and dietary routes of exposure. However, a sensitivity analysis will be performed to determine the impact of including an estimate of inhalation background exposure.

subjected to a complex Monte Carlo analysis to simulate the impact of uncertainty and variance on key inputs that factor into the extrapolation of exposure metrics to µg/kg-day dose; for example, some studies might report arsenic concentrations for a particular route of exposure (e.g., drinking water) but not consider contribution from other sources such as dietary or inhalation exposure; other studies might report arsenic exposure concentrations from a particular source (e.g., a community water supply) rather than individual exposure levels. Furthermore, these source concentrations might be estimated from samples taken over a limited period or a single time point and extrapolated to lifetime exposures. Therefore, studies where exposure was determined on the individual level are assumed to introduce less uncertainty to associations between health effects and inorganic arsenic than those that used community measures of exposure.

Aspects of exposure characterization are relevant to the use of such data in dose-response analyses. For estimating total daily exposure, the National Research Council (NRC) indicated that exposure routes (i.e., drinking water, diet, air, smoking) should be characterized, preferably using probabilistic approaches (NRC, 2013). In response to this recommendation, EPA has qualitatively and, where possible, quantitatively delineated between routes of exposure and considered information provided by biomarkers of exposure. Studies conducted on U.S. populations and other populations (e.g., Taiwanese, Bangladeshi) have been evaluated for hazard identification and a determination will be made on whether an adjustment in estimated dose-response behavior in the U.S. population is warranted.

5.2.1. Dose-response modeling approaches

Multiple separate dose-response analyses will be considered for the inorganic arsenic assessment. Dose-response analyses will be performed on health outcomes with *robust* or *moderate* evidence reporting an association between arsenic exposure and effect, or those considered important for Agency benefit-cost analyses. As previously discussed, EPA's dose-response analysis approach follows Agency guidelines considering recommendations from NRC (NRC, 2013) and (NASEM, 2019), which include:

- accounting for total inorganic arsenic from all exposure routes,
- focusing on epidemiological studies with low-to-moderate arsenic exposure (<100 µg/L in drinking water),
- describing dose-response down to background (1–5 µg/L total urinary As),
- using observed data, when possible, with modest extrapolation to background, if necessary,
- considering nonlinear and linear dose-response models, considering meta-analyses for well-studied, priority health outcomes,
- considering how MOA information can inform dose-response analyses, and

- estimating doses associated with a probabilistic risk (e.g., extra risk with 95% confidence) of effects with a defined level of severity.

The dose-response modeling approaches considered for this Toxicological Review have been presented in a series of publications (Hobbie et al., 2020); (Mendez et al., 2020); (Allen et al., 2020a); (Allen et al., 2020b) and are described in Table 5-4 as three approaches. When study data are modeled by EPA using one of the approaches summarized in Table 5-4, the first step is the conversion of all exposure information to a single intake dose metric (µg/kg-day) and the adjustment of responses to account for covariates. If study data are sufficient, the first approach attempted will use a meta-regression multiple study analysis. A single study or meta-regression can be performed using the exposure metric (e.g., µg iAs/L drinking water) and regression coefficients generated by study authors and converting the resultant POD from the study exposure metric to a µg/kg-day dose metric. However, this limits the number of studies that can be combined to those that use the same dose metric and requires information that is not available for key arsenic studies. If warranted (e.g., for the purposes of determining model uncertainty associated with a given study data set), model averaging methods may be applied. If those methods are not feasible, appropriate, or applicable, an analysis of individual studies using published modeling results or EPA modeling using a traditional benchmark-dose approach in which a single "best" model is chosen in accordance with EPA guidelines.

As illustrated in Table 5-4, the model averaging and meta-regression analyses will differ from the more simplified single study, single model dose-response analyses regarding the types of study data analyzed (individual instead of grouped data), the numbers of studies evaluated (use of multiple study meta-analyses, where feasible), and the complexity of the models employed.

Table 5-4. Summary of proposed inorganic arsenic dose response methods

Dose-response element	Approach	Meta-regression	Model averaging	Single study, selected EPA model
Type of study data	Grouped exposure, outcome, or both	✓	✓	✓
	Multiple study data sets (meta-analysis and similar)	✓		
Dosimetry	Use estimates of intake dose that considers exposures from multiple routes obtained from multiple published sources	✓	✓	✓
	Use biomarker data	✓	✓	✓
	Intraconversion of intake/biomarker metrics based on empirical data, physiologically based pharmacokinetic models	✓	✓	✓
Dose-response model forms	Standard parametric models (Poisson regression, benchmark dose-type models, etc.)		✓	✓

Dose-response element	Approach	Meta-regression	Model averaging	Single study, selected EPA model
	Complex parametric and nonparametric models (random effects, etc.)	✓		
Dose-response modeling methods	Conventional (primarily maximum likelihood estimate)		✓	✓
	Bayesian (Markov Chain Monte Carlo)	✓		
	Model averaging		✓	
Output risk metrics	Points of departure, risk-specific doses, low-dose slope factors, or equivalent			✓
	Model-based risk estimates	✓	✓	
	Fully probabilistic risk estimates	✓		
Uncertainty and variability	Risk for subpopulations based on quantitative estimates of sensitivity (absorption, distribution, metabolism, excretion, etc.)	✓	✓	✓
	Probabilistic modeling of exposure, pharmacokinetic, and prior distribution uncertainty as supported by data	✓		
Low-dose extrapolation	Within range of study data			✓
	Statistical confidence limits on predicted risks	✓	✓	

The meta-regression dose-response analysis will use Bayesian analyses based on data from multiple studies, where possible, to derive fully probabilistic risk estimates. In analyzing bladder cancer, lung cancer, diseases of the circulatory system (DCS) and diabetes, EPA will apply model averaging, meta-regression and single-study, best model dose-response analyses. The hierarchical Bayesian approach encompassed in the meta-regression method will be the focus of the assessment.

Study quality is still an important consideration for a meta-analysis but, due to the statistical benefits of combining data from multiple studies, it is not necessary for studies considered in a meta-analysis to be as high quality as studies considered for an individual study analysis. In particular, an individual study's statistical precision (e.g., study size or number of dose groups) is less important as a key reason for considering the combination of study data in a meta-analysis is to improve overall precision. In judging whether studies are suitable for combining in a meta-analysis, EPA will also consider whether the endpoint is a measure of the same health outcome and whether the exposure metric is or can be converted to the same dose metric.

In addition to the considerations summarized above, statistical issues can affect dose-response modeling for individual data sets (see the *Benchmark Dose Technical Guidance* § 2.1 and Figure 2A for further detail) (U.S. EPA, 2012). While studies with increased responses over the referent/control group at more than one exposure/dose levels are preferred, studies with no or nonmonotonic exposure-response relationships are not necessarily excluded from the analysis (U.S.

EPA, 2022). A diminished effect at higher exposure levels might be satisfactorily explained by factors such as competing toxicity, saturation of absorption or metabolism, exposure misclassification, or selection bias see 2.3.6, U.S. EPA (2012). Studies showing null or very weak effects but judged consistent with studies showing stronger effects (based on differences in study design such as exposure levels or sensitivity) generally would not support their own toxicity value derivations but are possibly appropriate for inclusion in meta-regressions or meta-analyses, with appropriate incorporation of the noted differences in study confidence evaluation or other attributes.

The logistic model EPA will use for modeling epidemiologic data in the meta-regression approach makes no assumption regarding the shape of the dose-response curve (linear vs. nonlinear) or whether a threshold exists in the dose-response relationship, meaning it can adequately describe threshold and non-threshold dose-response curves. However, it does not allow for a change in the dose-response direction (e.g., "J"- or "U" -shaped dose-response curves). Also, using multiple epidemiologic studies consisting of different populations and life stages with different levels and magnitudes of susceptibilities tends to linearize the dose-response relationship in the low-dose region. To investigate the potential impact on EPA's meta-regression analysis to the use of models that allow for non-monotonicity, EPA performed a Bayesian hierarchical modeling sensitivity analysis using fractional polynomial models and "double Hill" models. The application of these non-monotonic models to EPA's epidemiology meta-regression datasets does not support the hypothesis that risk do not exist or decrease with increasing dose for any levels of exposure at or above the current background exposure level. Details of this sensitivity analysis are described in Appendix C, Section C.1.1.5.

A distinguishing feature of the meta-regression approach is the use of Bayesian (Markov Chain Monte Carlo) approaches to generate distributional outputs based on the data and the assumed prior probabilities for models and distributions of model parameters. Depending on data availability, EPA will analyze the impact of latency, potentially sensitive subgroups, children, who have greater intake of water and food per body weight than adults, or groups with higher nondietary levels of exposure (e.g., smokers). EPA will also use (where possible or needed) empirical data or physiologically based pharmacokinetic models to compare studies that present risks as a function of exposure with those that present risks as a function of biomarkers, such as urinary arsenic. Additionally, the hierarchical Bayesian method used for the meta-regression allows for the analysis of case-control and cohort studies, as well as low-dose and high-dose studies, simultaneously. Finally, an approach to estimate the lifetime risk of developing the disease of interest due to inorganic arsenic exposure will be utilized in the assessment. This approach, called the life table approach, estimates the probability that an individual, exposed over an entire lifetime, will develop the disease of interest, accounting for the background probability of developing the disease (i.e., probability of developing the disease at the background level of inorganic arsenic exposure). This life table-based extrapolation used age-stratified U.S. all-cause

mortality and disease-specific morbidity and mortality statistics, and as such is conceptually a method to extrapolate risks estimated in the studies used in the meta-regression to lifetime risks in the general U.S. population. With respect to smoking, EPA will account for the potential increases in arsenic exposure via cigarette smoke in two stages of the meta-regression: (1) as part of the probabilistic dose conversions and (2) when estimating the U.S. background exposure level to arsenic for use in the life table extrapolations.

5.2.2. Extrapolation for dose-response analyses

In addition to using observed data to characterize dose-response relationships, NRC also recommended limited extrapolation of fitted models to within an order of magnitude of the observed data. Model choices planned for the current analysis allow for nonlinear or threshold phenomena, as supported by the data. NRC further recommended characterizing dose-response relationships down to (but not necessarily below) background levels, estimated to be 1–5 μg/L inorganic, monomethyl, and dimethyl arsenic forms of arsenic in urine for U.S. populations. NRC indicated that the risks below background concentrations should be characterized to the extent feasible but also assumed the needs of risk assessors would be met if risk can be characterized down to background concentrations. Extrapolations in the Toxicological Review will be informed by these recommendations, and a life table approach will be used in conjunction with the Bayesian meta-regression to extrapolate risks to the general U.S. population. The life table analysis will use current information on all-cause mortality, and the lifetime incidence and mortality rates of the diseases under consideration.

5.2.3. Sensitivity analyses and other methods for identifying susceptible populations/life stages

EPA has identified pregnant women, fetuses and developing children as sensitive subpopulations and has performed dose-response analyses on available studies of pregnancy outcomes and neurodevelopmental, neurocognitive effects. In addition, when possible, EPA has conducted sensitivity analyses to quantify the impact of uncertainty/variability in key factors such as exposure characterization, nutritional status, model and study choice, susceptible populations, life stage, and covariates.

The database of long-term epidemiological studies of arsenic is extensive. Therefore, human data were used as the primary source of dose-response information. Animal studies and mechanistic information provide additional support. Observed variability in human (particularly U.S. population) responses, studies involving sensitive subpopulations including early-life exposures, and quantified variability in relevant pathological events (e.g., metabolism) that are key to the mode of action for a given arsenic endpoint were considered for estimating risk, and risk confidence intervals, for U.S. populations.

6. PROTOCOL HISTORY

Release date: 5/28/2019

April 2023: The characterization of newly identified studies from recent literature search updates will focus on EPA's judgment of whether the studies would have a material impact on the conclusions (i.e., identified hazards or toxicity values) in the external review draft.

August 2022: The PECO was refined to further prioritize health outcomes based on hazard judgment, RRB, and potential use for benefit-costs analysis by program offices. These outcomes include bladder cancer, lung cancer, DCS, diabetes, pregnancy and birth outcomes, and neurodevelopmental effects. The strength-of-evidence judgement of human evidence of a causal association for pregnancy outcomes was also updated to moderate from robust based on recent updates to the IRIS Handbook (U.S. EPA, 2022). Dose-response modeling methods were updated to include the estimation of a zero-dose response, which allowed for derivation of traditional reference dose (RfD) and cancer slope factor (CSF) values that reflect risk above a zero-background dose.

March 2021: Comments on this protocol were provided in the public docket (see Docket ID: EPA-HQ-ORD-2012-0830 for detailed comments) during a 30-day public comment period from May 28, 2019, to June 27, 2019. Individual comments were provided across a range of stakeholder groups. We thank the public commenters for their constructive and informative reviews. All comments were considered, and the updated methods applied, during development of the IRIS inorganic arsenic assessments. A summary of the public comment topics and corresponding updates is provided in Table 6-1, and other updates to the protocol are described below. Editorial comments were accepted unless otherwise indicated.

Table 6-1. Topic areas of public comments on the protocol and how comments were addressed in the updated protocol (generally ordered based on descending number of comments on the topic areas)

Topic areas raised by commenter(s)	Protocol updates and responses
Study Evaluations (See Protocol Sections 2.3.1, 3.1, 3.2, 3.7 and 5.1, and Appendix C)	
Summary of comments on which studies will undergo risk of bias evaluations: Clarify whether studies of all health outcomes would receive risk of bias evaluations and whether it is appropriate to exclude studies (e.g., novel statistical or meta-analyses) that "…do not contain original data, such as scientific literature reviews."	Risk of bias evaluations were initially conducted on all health outcomes as tiered by the NRC (see Section 3.1.) The latest screening efforts focused only on bladder cancer, lung cancer, DCS, diabetes, pregnancy outcomes, and neurodevelopmental. PECO has been updated in Section 3.2. As described in Section 3.7, studies that do not contain original data are considered "potentially relevant supplemental materials." Relevant primary studies included in

Topic areas raised by commenter(s)	Protocol updates and responses
	meta-analyses and reviews were considered in the systematic reviews conducted by EPA.
Summary of comments on the risk of bias criteria: Some criteria that appear to have been drawn from other assessments are not relevant to iAs.	See Appendix C for risk of bias questions and arsenic specific clarifications.
Summary of comments on use of DRAGON: DRAGON extraction process should be explained, and the DRAGON process should be made available for public review.	DRAGON is no longer available, and data has been migrated to HAWC and can be found here: Add URL. Clarified in Section 3.10.
Summary of comments on the relevance of animal studies: Some commenters suggested that animal studies should not be deemphasized (principally for the sake of MOA considerations). Others suggested that EPA should be more critical of the mouse studies conducted by Waalkes and colleagues.	As discussed in Section 2.3.1, animal and mechanistic evidence has been considered as supplemental evidence in the EPA assessment, an approach supported by the NRC (NRC, 2013) and consistent with assessments by others (ATSDR, 2007); (EFSA, 2009); (TCEQ, 2017).
Summary of comments on deemphasizing ecological studies: Ecological studies should be included among the information sources of the risk assessment.	Ecological studies are included for consideration and evaluated.
Summary of the comments on a studies relevance to US populations: Factors such as nutritional status can impact susceptibility of population subgroups to iAs exposure.	Risk factors, including nutritional status, are considered (see Table 3-5).
Summary of comments on Table 5-1, Rating criteria for inorganic arsenic exposure or dose response data sets for prioritizing studies for dose response analysis: Commenters requested more detail and specifics on the rating criteria and how they will be used to include or exclude studies.	Clarifying text has been added to Section 5.1 and Tables 5-1 and 5-2 have been updated.
Health Outcome Weight of Evidence (See Protocol Sections 2.3.1 ,3.1.11 and 3.2, and Tables 2-2 and 3-1)	
Summary of comments on the need for clarification of the systematic review process: Comments varied but generally focused on the need for better clarification of how the robust, moderate, or slight classifications were assigned, suggesting that the confusion stems from the use of a complicated blend of OHAT, GRADE, and EPA guidance.	Section 3.1.11 includes description of the considerations that inform judgments regarding the strength of the human evidence and framework for evidence judgments from studies in humans.
Summary of comments on use of other reviews to determine weight of evidence for some health outcomes: This approach will result in evidence analysis being unevenly conducted across health outcomes.	Reviews were not used alone in determining strength of evidence judgments. NRC tiering and conclusions from other health agency assessments were also considered, See Table 2-2.
Summary of comments on the impact of new studies: new studies (e.g., new prostate cancer epi studies, in vitro and animal studies were identified) should be considered and may change evidence classifications and dose-response analyses.	As noted in Table 3-1, a broad outcome search strategy was retained during the different phases of outcome prioritization. Epidemiological studies on other health outcomes not prioritized are tagged during screening to monitor for new studies that may affect the problem formulation decisions described above.
MOA (See Protocol Sections 2.3.2 and 5.2.2, and Appendix A)	
Summary of comments on the human, animal and in vitro evidence for a threshold and inverted dose-response: Human, animal, and in vitro data should be used to support the adoption of a dose-response threshold. Thus, the same RfD-	Numerous, high quality low dose epidemiological studies of arsenic's association with multiple health effects exist. As described in Section 5.2.2, the application of non-monotonic models to EPA's epidemiological meta-regression datasets

Topic areas raised by commenter(s)	Protocol updates and responses
type approach should be used for cancer and noncancer outcomes. EPA should also discuss MOA and empirical evidence that suggests the possibility of an adaptive or protective response at low doses.	does not provide evidence for no risk or decreasing risk with increasing dose for any levels of arsenic dose at or above the current background dose level. In addition, EPA has performed a thorough analysis of MOA evidence from animal and human studies (Appendix A) and has concluded that the possibility of risk at low doses above background cannot be definitively ruled out from the existing database of evidence.
Summary of comments on NRC/NAS recommendations regarding MOA: NRC recommendations to the EPA include a "clear call to examine low dose issues in terms of modes of action and not simply to extrapolate from high doses in epidemiology studies." EPA's approach is not consistent with NRC/NAS recommendations.	Appendix A presents a case study to address the feasibility of using MOA and mechanistic data to address the feasibility of using this information to inform dose-response modeling with respect to the shape of the curve, particularly in the low dose region. Ultimately, the MOA analyses were not considered more suitable than the epidemiological studies. The NRC also acknowledged uncertainty about whether such analyses would be feasible without further research (NRC, 2013). See Section 2.3.2 and Appendix A.
Summary of comments on EPA's dose-response assumptions: EPA assumes, or allows for, a linear dose-response that is not relevant because iAS dose not react with DNA. EPA should provide an alternative analysis that uses MOA information to inform dose-response shape/extrapolations.	Appendix A presents a case study to address the feasibility of using MOA and mechanistic data to address the feasibility of using this information to inform dose-response modeling with respect to the shape of the curve, particularly in the low dose region. Ultimately, the MOA analyses were not considered more suitable than the epidemiological studies. The NRC also acknowledged uncertainty about whether such analyses would be feasible without further research (NRC, 2013). See Section 2.3.2 and Appendix A.
Summary of comments on possible iAs MOAs: All the possible modes of action proposed for arsenic have thresholds, and it is widely accepted that the biological effects of arsenicals are due to a threshold reaction of trivalent forms with sulfhydryl groups, leading to cytotoxicity and regeneration. Consideration should be given to the role of digestive tract microbiome in the dosimetry/toxicity of As.	In addition to cytotoxicity and regenerative proliferation, other MOAs are likely involved in both cancer and noncancer outcomes. These include reactive oxygen species (ROS) generation and oxidative stress responses, As(III) binding to thiol groups and inhibition of key enzymes, As(V) inhibition of oxidative phosphorylation, cell cycling and damage repair impairment, epigenetics, and endocrine disruption. See Appendix A.
Summary of comments on the interpretation of EPA Cancer Guidelines: EPA Cancer Guideline recommendations are not, as the Protocol suggests, most relevant when human data/evidence is limited or unavailable. MOA should be considered for each endpoint associated with iAs exposure to inform dose-response extrapolations even when human low dose data is available.	Specific to inorganic arsenic, a MOA analysis was considered less effective for hazard characterization given the abundance of epidemiological evidence, including at low levels of exposure, and recognition that data from animal studies of inorganic arsenic are of limited applicability for dose response analysis in human health risk assessment. See Appendix A.
	Toxicokinetic/PBPK Model (See Protocol Section 4)
Summary of comments on suitability of the (El-Masri and Kenyon, 2008) iAs PBPK model: The iAs PBPK model is a human male model and is for the oral route of exposure only. Revisions to the model will be necessary to evaluate exposure in different subpopulations (including pregnant and nonpregnant females) and to evaluate inhalation exposure for development of an RfC or IUR.	We agree with the commenter that the (El-Masri and Kenyon, 2008) model was constructed for an average human. The model can be used for an average non-pregnant female since the calibrations for the model were conducted using human data that were both male and female. However, expanding the model scope to include pregnant females will require calibration, parameterization, and evaluations that are dependent on the availability of human data. The scope of the inorganic arsenic assessment currently is oral exposure.

Topic areas raised by commenter(s)	Protocol updates and responses
Summary of comments on sensitivity analysis of the (El-Masri and Kenyon, 2008) iAs PBPK model: A systematic sensitivity and uncertainty analysis is needed for each parameter in the model is needed to understand the most sensitive parameters in the model and whether adequate data are available to support the parameter values used in the model.	Most of the parameters used in the model were either calculated or directly obtained from literature. The publication of the model (El-Masri and Kenyon, 2008) illustrates the methods used to justify the use of these parameters. Sensitivity analysis was performed to investigate the impact of some parameters that are model-fitted for cumulative As and its metabolites in urine (Figure 5 in (El-Masri and Kenyon, 2008)). Additionally, the approach for estimating overall model parameters started with least number of parameters for model simulations using urine data for DMA and progressively moving to more complex models for MMA and iAs. We agree with the reviewer to the usefulness of uncertainty and variability analysis, however applications of these methods depend on the availability of large data sets for humans.
Populations, Exposures, Comparators, and Outcomes (PECO) Criteria (See Protocol Section 3.2)	
Summary of comments on PECO content: Animal and mechanistic data are PECO relevant and should not be considered "supplemental material." Other important elements, like ADME, should be added to Table 3-1.	The PECO for the inorganic arsenic assessment has evolved over time and is based on refined scope that was informed by prior science discussions with the NRC, EPA program and regional offices, and other stakeholders. ADME is considered "potentially relevant supplemental materials."
Summary of comments on PECO clarity: The PECO criteria are too vague (e.g., what is a "reference population with no detectable exposure or exposure to lower levels of iAs?")	PECO for Comparators has been revised for clarity: A comparison or referent population exposed to lower levels (or no exposure/exposure below detection limits) of inorganic arsenic, or exposure to inorganic arsenic for shorter periods of time, or cases vs. controls. See Table 3-1.
Dose-Response – RRB Screening Approach (See Protocol Section 5.1)	
Summary of comments on RRB derivation: Comments varied, but focused on the need for clarification on the specific (e.g., DCS) health outcomes that were considered, study selection (Table 5-1), how background estimates were obtained, the dose-response modeling process, RRE derivation, RRB derivation,	Clarifying text has been added to Section 5.1, including tables 5-1 and 5-2. As explained in Section 5.1 and Table 5-3, EPA focused on health outcomes with *Robust* and *Moderate* evidence for the RRB screening. The purposes of this preliminary screening (Hobbie et al., 2020), which includes comparing health outcomes with respect to potency and number of studies, would not be served by excluding high exposure studies of moderate quality.
Dose-Response Bayesian Meta-Regression Approach (See Protocol Section 5.2)	
Summary of comments on conversion of study exposure metrics to common dose: Provide more details on the Monte Carlo uncertainty analysis and exposure factors considered. Do not use author estimates of intake as they are prone to self-reporting bias, i.e., those with the disease (or their relatives) may be more likely to recall drinking greater quantities of water than those without the disease.	As state in Section 5.2.1.4, details on how variability and uncertainty were considered and addressed in the dose response analyses for inorganic arsenic are provided in Section 3.3 of the iAs assessment. With respect to the estimation of iAs intake, it should first be noted that EPA believes that associating responses with estimates of actual iAs oral intake is a key component of assessing iAs risk, particularly in US and other 1st world populations that have a relative abundance of alternative hydration sources and are generally better informed and educated regarding the

Topic areas raised by commenter(s)	Protocol updates and responses
	existence of and consequences associated with drinking from contaminated sources. Second, EPA believes that study-specific author estimates of oral intake, often from individual surveys or measurements, are more reliable than default assumptions, and that the potential for recall bias is not an overriding concern. In fact, one of the largest and best studies performed in the US, the NCI iAs bladder cancer study (Baris et al., 2016), determined that ORs did not change when subjects that said they had made a major change in drinking water consumption during their adult life were excluded, "suggesting that recall bias is not a major concern." They also found that subjects that were unaware of the potential harmful effects of their drinking water had higher ORs than subjects that were aware and commented that "if recall bias was seriously impacting our findings, it is unlikely that unaware subjects would have a more pronounced water intake effect than aware subjects."
Summary of comments on the proposed modeling methods: Comments varied but focused on the need to consider MOA to inform model shape, prioritizing low exposure data, sensitive subgroups (e.g., nutritionally deficient, smokers, etc.), flexible (including nonmonotonic) models, prior sensitivity (for Bayesian models), and heterogeneity across studies. Meta-analyses have a "tendency toward linearization of the apparent dose-response (Crump, 2005) (Rhomberg et al., 2011). Methods that evaluate U shape, such as those used by Krewski et al., should be considered. Section 5 should give more details on the justification for the models and approaches chosen.	As requested, additional detail has been added to the Section 5.2 discussion of EPA's dose-response modeling approaches, including a description of EPA's sensitivity analysis involving the application of flexible models that can take on U-shape (i.e., non-monotonic) dose-response curvatures. The MOA section (Section 2.3.2) has also been expanded (see also the response above in this table regarding the use of MOA for informing dose-response).
Summary of comments on the derivation of slope factors from the modeling results: EPA should not presume that there is not a threshold for cancer and noncancer effects down to background. When there is believed to be a threshold, an RfD should be derived for both cancer and noncancer endpoints. Also, how will EPA determine if the dose-response is "sufficiently linear" for the derivation of slope factors?	EPA will derive an RfD when there is believed to be a threshold for both cancer and noncancer endpoints. If a slope factor is derived from the results of the EPA's meta-regression analyses, EPA will apply appropriate statistical methods to determine whether the results are statistically different from linearity. If necessary, EPA will specify the range of doses for which the slope factor (linearity) should and should not be applied.
Literature Identification (See Protocol Sections 3.3 and 3.4)	
Summary of comments on studies identified: several articles in the HERO database that would appear to be important for this analysis were neither used nor in the Reference section, possibly due to EPA's too limited PECO criteria.	This document is an updated problem formulation and protocol, not the actual assessment. This document describes the refined scope and methods that will be used in developing the assessment. Therefore, it is not expected that the reference section in this document would include all studies considered in hazard identification and dose-response analyses.
Summary of comments on "pilot phase" used to "calibrate screening guidance:" More information is needed to determine how IRIS assessed this pilot program for success.	After the pilot phase, screeners met to discuss results of screening and refined screening guidance, as needed, based on pilot phase results.
Summary of comments on search strategy: USEPA's use of machine learning/clustering methodology needs to be explained in more and clearer detail. Searches (e.g., PubMed) are not reproducible based on the information provided in	Section 3.4 describes the use of machine learning to prioritize studies for screening. As discussed, the initial literature search was comprehensive, and clustering was used to identify references most likely to contain data relevant to hazard

Topic areas raised by commenter(s)	Protocol updates and responses
the protocol document. Also, why were organic forms of As included and Arsenic Trioxide excluded?	identification. Clustering methodology is described in (Varghese et al., 2017). As stated in the PECO, arsenic trioxide was excluded because chemotherapeutic agents are not within the scope of this review. Organic forms are not included. The PECO is for an assessment of inorganic arsenic.
Other Comments (See Protocol Sections 3.2 and 5.2.1.2)	
Summary of comments on routes of exposure the iAs assessment will cover: The focus of the current scoping is unclear as to whether it includes oral exposure only or oral and inhalation exposure. It is unclear whether and how inhalation risk estimates would be developed.	The current scope is on oral exposure. Inhalation risk estimates will not be developed. This has been further clarified in Table 3-1 under" Exposures."
Summary of comments on evaluating risk at background exposures: Are risks associated with background going to be considered acceptable or will they be considered unacceptable? Will USEPA consider some level of exposure above background acceptable? If so, what procedures will be used to determine that level? Moreover, given that natural background exposures vary substantially across the United States, how will USEPA establish background concentrations of inorganic arsenic in relevant environmental media?	The contribution of background iAs exposure to background risk and has not been estimated by EPA for any health effect. Clarification has been added to Section 5.2.1.2 regarding EPA's characterization of the iAs dose-response down to background concentrations. As discussed in Section 5.2.1.2, the NRC (2013) has stated that it "…assumes that the needs of assessing health risks can be facilitated by characterizing dose–response relationships down to the background concentrations by using observed data." Consistent with this assumption, EPA has estimated extra risk from iAs doses above background doses. EPA will derive noncancer toxicity values (i.e., toxicity values that may be comparable to a reference dose; RfD) that are estimates of the total chronic dose (including background iAs dose) to U.S. populations (including susceptible subgroups and/or life stages) that are likely to be without an appreciable extra risk above the background risk (set to the background iAs dose) of adverse health effects over a lifetime (Section 4.6).

1

APPENDIX A. ANALYSIS OF MODES OF ACTION COMMON TO MULTIPLE HEALTH EFFECTS

A 1. BACKGROUND

EPA defines mode of action (MOA) as "a sequence of key events and processes, starting with the interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation [or other adverse outcomes]" (U.S. EPA, 2005a). EPA's Guidance drew upon earlier publications by World Health Organization's (WHO) International Programme on Chemical Safety (Sonich-Mullin et al., 2001) and the Framework for Human Relevance Analysis of Information on Carcinogenic Modes of Action, published by members of the International Life Sciences Institute Risk Science Institute (ILSI RSI) (Meek et al., 2003). These principles are outlined in section 2.4: MOA Framework Guidelines of the EPA Cancer Guidelines document and provide guidance for the development of MOA analyses. The guidelines state that "mode of action conclusions should be [are] used to address the question of human relevance of animal tumor responses, to address differences in anticipated response among humans, such as between children and adults or men and women; and as the basis of decisions about the anticipated shape of the dose-response relationship." (See Sections 2.4.2.2 and 2.4.3.4 of (U.S. EPA, 2005a)). In addition, regarding conclusions about the hypothesized MOA Action, the guidance further states: "[conclusions about the Hypothesized Mode of Action should address the issues listed below.] For those agents for which the mode of action is considered useful for the risk assessment, the weight of the evidence concerning mode of action in animals as well as its relevance for humans would be incorporated into the weight of evidence narrative (Section 2.5)."

The IRIS Program routinely conducts MOA analyses to inform hazard identification and dose-response analysis but having a complete understanding of MOA is not required to develop hazard conclusions or toxicity values (U.S. EPA, 2005a). In the case of arsenic, the NRC recommended EPA conduct MOA analyses to facilitate understanding of exposure-response relationships and interindividual variabilities for health outcomes where extrapolation to below the observed range may be necessary. However, the NRC also recognized that it was not clear whether such an analysis would be feasible.

A traditional MOA analysis was considered less impactful for hazard characterization given the abundance of epidemiological evidence, including at low levels of exposure, and recognition that data from animal studies of inorganic arsenic to be unreliable for dose-response analysis in human health risk assessment (ATSDR, 2007). These data are only considered when human data was lacking (ATSDR, 2007). Similarly, EFSA concluded that results of toxicity studies in animals do not provide suitable basis for risk characterization (EFSA, 2009). EPA concurs with these

interpretations. Similarly, this approach is consistent with the Texas Commission on Environmental Quality (TCEQ) plan (TCEQ, 2017) on adopting an oral cancer slope factor (CSF) for inorganic arsenic based on the meta-regression analysis conducted by (Lynch et al., 2017a) that focuses on data solely from epidemiological studies. In addition, in recent years, many new epidemiological studies on the toxicity of inorganic arsenic have been published for a broad range of cancer and non-cancer outcomes, including large-scale longitudinal cohort studies, case-control studies, and cross-sectional studies. Due to the extensive amount of epidemiological data and the difference in susceptibility between animals and humans (with humans being more sensitive to arsenic), hazard identification and dose-response analysis for this assessment will rely predominantly on human data.

This Appendix describes the analyses conducted by EPA to characterize MOAs associated with arsenic exposure, focusing on MOAs that may be common to multiple adverse health effects versus tissue specific descriptions. In addition, at the recommendation of the NRC, extensive MOA analysis was conducted for bladder cancer to better understand human variability and the possible use of mechanistic data to inform low dose extrapolation. As will become evident, complete, recognized MOAs for any of the hypothesized bases for iAs induced disease are incomplete, poorly populated with key events, and/or nonspecific. Further, while the. The NRC recommended MOA evaluation provided additional support by identifying arsenic-specific mechanisms and risk modifiers likely to increase risk of human bladder cancer. However, the impact and utility of mechanistic information on dose-response analyses was minimal because much of the primary MOA evidence is based on in vitro studies at doses that pertain to unrealistically high physiological concentrations (~100 μM). This raised concerns about the applicability of in vitro studies to inform low-dose effects. Further, the MOA evidence base does not contain studies that examine and or establish an MOA for arsenic induced disease(s) in a sufficiently dose responsive manner to be able to inform the shape of a dose response at very low doses. Together, these factors prevent a critical evaluation of dose response relationships, particularly in the low dose region.

The mechanisms of arsenic associated disease induction are complex. The categorization of MOAs below is primarily taken from the NRC 2013 report (NRC, 2013). Evidence for genotoxicity mechanisms of action are discussed under the various MOA categorizations below including ROS Generation and Oxidative Stress Responses, Sulfhydryl Binding, and Epigenomics. Impaired DNA repair is associated with iAs-induced tumorigenesis and other adverse effects and is discussed under the "ROS Generation and Oxidative Stress Responses" and "As(III) Binding to Thiol Groups and Inhibition of Key Enzymes" MOAs. Complex individual MOAs or biochemical interactions (e.g. sulfhydryl binding and inhibition of phosphorylation) are involved in the pathogenesis of multiple diseases. Below when presenting MOA analyses, we describe diseases that are associated with a given MOA, this was not done for the two inorganic arsenic biochemical interactions, sulfhydryl binding and inhibition of phosphorylation.

Evidence suggests arsenic initiates a cascade of intracellular events that culminate in aberrant gene expression changes, cell growth dysregulation, proliferation, differentiation, and antioxidant defense (Ren et al., 2015); (Medeiros et al., 2012); and reviewed in (Bustaffa et al., 2014). For example, global gene expression is substantially altered in individuals following arsenic exposure (Andrew et al., 2008); (Bourdonnay et al., 2009); (Bustaffa et al., 2014). Further, characterization of genomic methylation suggests that dysregulation of epigenetic processes could contribute mechanistically to observed changes in iAs related gene expression and toxicities. Both global and focal deregulation of genomic methylation have been implicated in arsenic-induced malignant transformation in vivo and in vitro (Benbrahim-Tallaa et al., 2005); (Chanda et al., 2006); (Jensen et al., 2009b). Arsenic exposure also has been linked to histone modifications in vitro and in circulating leukocytes collected from chronically exposed humans (Jo et al., 2009); (Zhao et al., 2010); (Chu et al., 2011); (Ge et al., 2013), and to altered microRNA expression and target gene expression (Ren et al., 2011a); (Wang et al., 2011a); (Li et al., 2012); (Shan et al., 2013) ; (Martínez-Pacheco et al., 2014); (Ren et al., 2015). The various MOAs appear interrelated and are likely to be involved in both cancer and noncancer outcomes. The MOAs and biochemical interactions described below may exhibit individual dose-response thresholds however, this does not imply the existence of a population dose-response threshold given individual differences in the threshold due to environmental or genetic factors (NRC, 2007). While incorporation of MOA data is not proposed for the dose-response methods in this assessment given the multiplicity of different possible MOAs, explicit consideration of inter-individual variability was incorporated into the dose-response analyses, in adherence to 2007 and 2013 NRC recommendations (NRC, 2013, 2007).

MOAs discussed in this appendix include:

- Interrelated MOAs and Biochemical Interactions
  - ROS Generation and Oxidative Stress Responses
  - As(III) Binding to Thiol Groups and Inhibition of Key Enzymes
  - Epigenomics
  - Cytotoxicity and Regenerative Proliferation
  - As(V) inhibition of Oxidative Phosphorylation (As(V) structural analog of phosphate)
  - Endocrine Disruption

In addition to these categorizations, studies presenting information on other potential MOAs (e.g., immune system surveillance, stem cell modification) were tagged during screening in literature search updates and are available in HERO. However, given the refined assessment focus on epidemiological studies for dose-response these studies were not further analyzed.

A.1.1. MODE -OF -ACTION ANALYSES

Hypothesized Mode of Action (MOA): Reactive Oxygen Species (ROS) Generation and Oxidative Stress.

Relevant Health Effects: Cardiovascular Disease, Diabetes, Liver Disease, Lung Cancer, Bladder Cancer, Neurotoxicity, Nonmalignant Respiratory Disease, Pregnancy Outcomes, Renal Disease, Skin Cancer, and Skin Lesions.

Arsenic has been shown to induce the formation of multiple ROS including: $O_2^{\cdot}$, H_2O_2, $^{\cdot}OH$, and $ROO^{\cdot}$ (Hu et al., 2020b); (Eblin et al., 2008); (Eblin et al., 2006); reviewed in (Shi et al., 2004); see Table A-1), namely through the mitochondrial transport chain via contributions from NADPH oxidase (Nox) and arsenic metabolism (Corsini et al., 1999); reviewed in (Shi et al., 2004) and (Hu et al., 2020b); (Hu et al., 2020a). Many studies have shown that free radicals contribute to persistent DNA damage and epigenetic alternations even after arsenic exposure ceases (Flora, 2011); (Wnek et al., 2009). Further, arsenic also indirectly contributes to ROS accumulation by disrupting antioxidant homeostasis (Sarath et al., 2014); (Hu et al., 2020b); (Tam et al., 2020). (Flora, 2011) postulate that in addition to oxidative stress from the disruption of the mitochondrial electron transport chain described above, that arsenic-induced oxidative stress arises from the impaired activities of SOD, catalase, glutathione peroxidase, glutathione S-transferase and glutathione reductase. Collectively, the net effect of these is direct and indirect contributions to elevated levels of intracellular ROS. ROS signaling is critical in cytokine response, growth factor response and the activation/inhibition of signal transduction pathways (i.e. MAPR/ERK pathway) and transcription factors that promote cellular proliferation and carcinogenesis such as NrF2, AP-1, HIF-1α and p53 reviewed in (Shi et al., 2004) and (Lau et al., 2013); (Li et al., 2014). Further increased intracellular ROS also lead to oxidative stress that damages DNA (Wnek et al., 2011); (Li et al., 2001); (Ding et al., 2005) also see Table A-1, and lipid peroxidation (Maiti and Chatterjee, 2001); (Flora, 1999); (Santra et al., 2000).

As discussed in the Preamble, mammalian metabolism of inorganic arsenic involves a cascade of oxidation-reduction reactions whose net results are (1) generation of trivalent methylated species, (2) the generation of reactive oxygen species (ROS), and (3) depletion of cellular thiols that are involved in maintaining cellular redox balance. Several adverse health effects following exposure to inorganic arsenic may thus result from events mediated by oxidative stress (Kitchin and Conolly, 2010); (Jomova et al., 2011); (Flora, 2011) (Figure A-1). The *molecular initiating event (MIE)* in this MOA is a topic of ongoing investigation but likely includes one of the following: (1) intermediate arsine species (e.g., dimethylarsine) react with molecular oxygen, (2) methylated arsenic species react with ferritin, (3) arsenite oxidizes to arsenate, and (4) inorganic arsenic interacts with complexes in the mitochondrial electron transport chain and/ or antioxidant enzymes (e.g., nicotinamide adenine dinucleotide phosphate-oxidase [NADPH oxidase]) (Flora, 2011); (Li et al., 2014).

While multiple MIEs are possible for this MOA, each one will result in a biochemical response that consists of perturbing the redox balance in the cell through (1) generation of ROS (e.g., superoxide, H_2O_2, hydroxyl radical), and (2) depletion of antioxidant defenses (e.g., glutathione [GSH], ascorbate, superoxide dismutase) (Kitchin and Conolly, 2010); (Jomova et al., 2011); (Flora, 2011); (De Vizcaya-Ruiz et al., 2009). As illustrated in Table A-1, a variety of markers of oxidative stress have been measured in in vitro cell systems at concentrations in the low µM range, and in animal studies in the low mg/kg/day ranges (0.5–1.7 mg/kg).

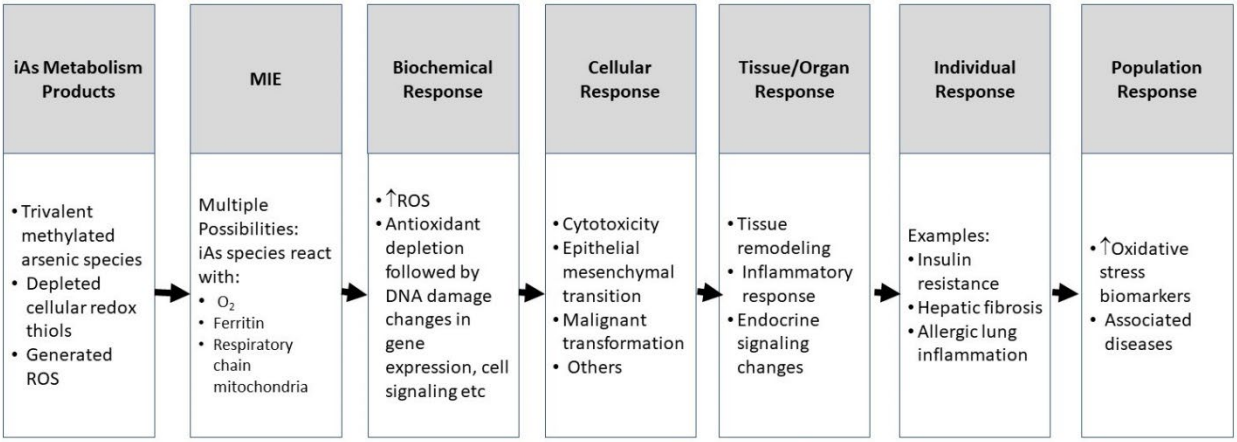


Figure A-1. Hypothesized mode of action for effects mediated by oxidative stress.

Abbreviations: Inorganic arsenic (iAs); molecular initiating event (MIE); reactive oxygen species (ROS)
See Summary Text and Table for references; Figure based on (Ankley et al., 2010).
Note: Figure shows key events from the initial molecular interaction through a possible population -level response. Arrows link each key event (e.g., individual responses lead to population responses), but do not necessarily link each specific example response (e.g., insulin resistance is not linked to all the diseases included in the table in Section A.3.). As the assessment development process moves forward, additional evidence may provide a better understanding of the key events in this MOA and the connections between them.

Numerous biochemical responses can occur within cells following the generation of ROS and or depletion of antioxidant defenses, including changes in protein expression, enzyme activity, lipid oxidation, DNA damage, gene expression and cell signaling (Table in Section 10.5). For instance, alterations in protein expression levels have been observed in multiple tissue types. While observations of increased protein expression levels related to antioxidant defense (e.g., Cu/Zn Superoxide dismutase [SOD], nuclear factor [erythroid-derived 2]-like 2 [Nrf2]) (Li et al., 2011);(Zheng et al., 2012);(Zhao et al., 2012) and DNA repair (e.g., DNA polymerase β) reviewed in (Snow et al., 2005) may occur across multiple cell types, other observations of elevated protein levels may be specific to specific cells (e.g., Platelet endothelial cell adhesion molecule [PCAM-1]) (Straub et al., 2008).

For many of the biochemical responses noted above, the concentration and duration of inorganic arsenic exposure, and subsequent redox imbalance, may influence the ultimate cellular response. Based on the literature reviewed, there appears to be a pattern of cellular responses (e.g., increases in antioxidant enzymes) at relatively low exposures (e.g., 0.1–1 μM iAs) with more pronounced adverse cellular responses at higher concentrations (e.g., decreases in DNA excision repair protein translation) (Snow et al., 2005). The exposure at which disruption of cellular homeostasis occurs varies greatly across cell lines, and thus the relevant concentration range that confers adaptive versus adverse cellular responses is a topic of ongoing research (Flora, 2011); (Clewell et al., 2011); (Gentry et al., 2010) reviewed in (Jomova and Valko, 2011). Similarly, acute or transient temporal increases in these proteins (e.g., elevated DNA repair enzyme activity at ≤ 48 hrs. of inorganic arsenic exposure, compared to basal activity levels after 72–120 hrs. exposure (Snow et al., 2005); (Eblin et al., 2008); (Medeiros et al., 2012);(Eblin et al., 2006);(Clewell et al., 2011);(Wnek et al., 2011); can suggest repair of induced DNA damage, while sustained elevation of these proteins suggests residual or unrepairable DNA damage. The observed sustained changes in DNA repair proteins seem to indicate that the damage to the cells was significant enough to cause these to begin to undergo apoptosis, pointing to ROS as a key player in this process (Shi et al., 2004); (Hartwig et al., 1997); (Wang et al., 1994); (Lynn et al., 1997); (Lee-Chen et al., 1993).

Separate from the consideration of exposure duration is the duration of a biochemical response that inorganic arsenic may elicit in a cell. The aspects of response duration are important to examine. Cellular responses such as brief elevations of ROS, can be a part of homeostasis and these differ from sustained and or unmitigated ROS levels that lead to oxidative stress and can adversely alter lipids, proteins, and DNA reviewed in (Devasagayam et al., 2004); and (Hu et al., 2020b). Evidence from in vivo and in vitro studies show that arsenic contributes directly and indirectly to the accumulation of ROS.

Hypothesized Mode of Action (MOA): Binding of As (III) to Thiol Groups and Inhibition of Key Enzymes

Relevant Health Effects: Multiple Outcomes

Inorganic arsenic binds to vicinal sulfhydryl groups in proteins and low -molecular -weight compounds such as amino acids and peptides (NRC, 1999) (see Figure A-2 below). It has been shown that inorganic arsenic can bind to various enzymes, including DNA repair enzymes and GSH metabolism-related enzymes, resulting in enzyme inhibition (Hu and Snow, 1998); (Snow et al., 1999).

More recently, it has been shown that iAs binds to zinc finger proteins containing cysteine motifs with vicinal sulfhydryls at in vitro concentrations ranging from 10–100 μM iAs and 50nM MMA (Wnek et al., 2011); (Zhou et al., 2011) (Kitchin and Wallace, 2005); (Ramadan et al., 2009) such as that found in the DNA repair enzyme, PARP-1 (Wnek et al., 2011); (Zhou et al., 2011). PARP-1 is a critical enzyme involved in the initiation of base excision repair following single strand

damage in DNA. (Wnek et al., 2011) showed that arsenicals (50 nM MMA^{III} in vitro) bind to sulfhydryls within the zinc finger motif of PARP-1, displace zinc and inhibit PARP activation. Others have reported analogous findings showing arsenite (10 µM in vitro) binds to cysteine rich motifs on proteins, such as that found in riboflavin, resulting in disruption of oxidative protein folding (Ramadan et al., 2009) Collectively, the evidence in the present database suggest that arsenicals, specifically arsenite (As^{III} and MMA^{III}), bind to different cysteine-containing proteins and inhibit and or disrupt their normal functions. More recently, observations were made at lower in vitro concentration of iAs, these concentrations would correspond to much higher hypothetical drinking water concentrations (i.e., 50 nM MMA^{III} corresponds to ~38 ppm iAs in drinking water)[12].

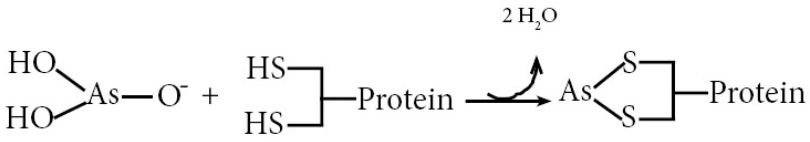


Figure A-2. Arsenic binding to vicinal sulfhydryls on proteins.

Hypothesized Mode of Action (MOA): Epigenetics[13]

Relevant Health Effects: Bladder cancer, skin cancer, skin lesions

As detailed below, several studies were identified that indicate epigenetic mechanisms may mediate some of the adverse health effects associated with exposure to inorganic arsenic (Figure 9-4). The depletion of and S-adenosylmethionine (SAM) may lead to one of the most well-studied of arsenic-associated epigenetic effects at the *biochemical response level*, namely, changes in DNA methylation patterns. As with arsenic 3+ methyltransferase (AS3MT), DNA methyltransferases (collectively, DNMTs) also utilizes SAM as a methyl donor. Therefore, reduced cellular SAM levels as a result of increased AS3MT activity could lead to reduced DNA methylation. Several studies have

[12] iAs conversions were performed by Dr. El-Masri using PBPK model (see Section 4). The model assumed a 2 L/day water drinking rate for a 70 kg individual.

[13]From (Argos, 2015): "DNA methylation is an epigenetic event with a hypothesized role in gene expression, development, and disease (El-Osta and Wolffe, 2000). In humans, methylation is typically of the DNA base cytosine, which is modified reversibly by adding a methyl group (–CH3) to its 5-carbon position (Herman and Baylin, 2003). This modification occurs on cytosines that precede a guanosine in the DNA sequence, referred to as the CpG dinucleotide. Short regions of 0.5–4 kb in length, known as CpG islands, are rich in CpG content. These islands are typically found in or near promoter regions of genes where transcription is initiated. In normal somatic cells, the vast majority of CpG dinucleotides in the genome are methylated, whereas CpG islands often remain unmethylated, allowing gene expression to occur. Whereas in disease pathways, this pattern of CpG methylation is thought to be disrupted, with increased methylation within promoter regions of genes causing abnormal gene silencing, in addition to global hypomethylation of genomic DNA, which promotes chromosomal instability, translocation and gene disruption (Esteller, 2007). Unlike CpG island regions, there is greater biologic variability in methylation of CpG dinucleotides in CpG shores (within 2 kb of a CpG island), CpG shelves (2–4 kb from a CpG island), as well as isolated CpG loci in the genome (Ziller et al., 2013). DNA methylation levels are influenced by various factors including genetic, environmental, and dietary factors (Mckay et al., 2012); (Siedlinski et al., 2012);(Philibert et al., 2012)."

found reduced levels of DNMT activity or expression in arsenic-exposed cell lines (Zhao et al., 1997); (Benbrahim-Tallaa et al., 2005); (Reichard et al., 2007). The observed changes in RNA expression levels suggest that factors in addition to SAM depletion may be responsible for changes in DNMT activity (Reichard and Puga, 2010).

Sufficiently reduced DNMT activity would likely inhibit cells' ability to maintain normal DNA methylation pattern and reduce the overall extent of DNA methylation. Global DNA hypomethylation after inorganic arsenic exposure has indeed been observed in a range of in vivo and in vitro studies (45–150 ppm iAs in vivo; 125 nM to 100 μM iAs in vitro) (Benbrahim-Tallaa et al., 2005);(Chen et al., 2004); (Chen et al., 2001);(Coppin et al., 2008); (Pilsner et al., 2012);(Reichard et al., 2007);(Sciandrello et al., 2004);(Xie et al., 2004);(Zhao et al., 1997) (Table A-4). As an example, treatment of human prostate epithelial cells, (RPWE-1) with 5 μM iAs for either 16 or 29 weeks, respectively resulted in hypomethylation (Coppin et al., 2008); (Benbrahim-Tallaa et al., 2005). Reduced DNMT activity and SAM depletion were observed in some, but not all, of these studies. A small number of studies have also reported global DNA hypermethylation in human populations at ranges of arsenic exposure ranging from (2–500 μg/L), (Majumdar et al., 2010); (Pilsner et al., 2007) and in animals at arsenic exposures ranging from (45–150 ppm) iAs (Chen et al., 2004); (Xie et al., 2004).

It has been suggested that the depletion of glutathione and SAM during cellular metabolism of inorganic arsenic species are important molecular initiating events (MIEs) of this MOA (Reichard and Puga, 2010); (Ren et al., 2011b); (Martínez et al., 2011). In addition, inorganic arsenic can also elevate levels of reactive oxygen species (ROS), which may in turn deplete SAM, in conjunction with, or separately from SAM depletion that results from inorganic arsenic methylation. Specifically, some evidence suggests that the depletion of glutathione (GSH) due to elevated oxidative stress results in the shunting of S-adenosyl homocysteine to replenish GSH, through the transsulfuration pathway, and away from the synthesis of SAM, inducing a shortage of methylation cofactors reviewed by (Reichard and Puga, 2010). Consistent with these findings and with multiple observations of GSH depletion, some investigators interpret the downstream epigenetic changes associated with inorganic arsenic exposure as mainly resulting from oxidative stress effects (Kitchin and Conolly, 2010); see Oxidative Stress MOA Summary, however, this interpretation of these observations has not been thoroughly investigated.

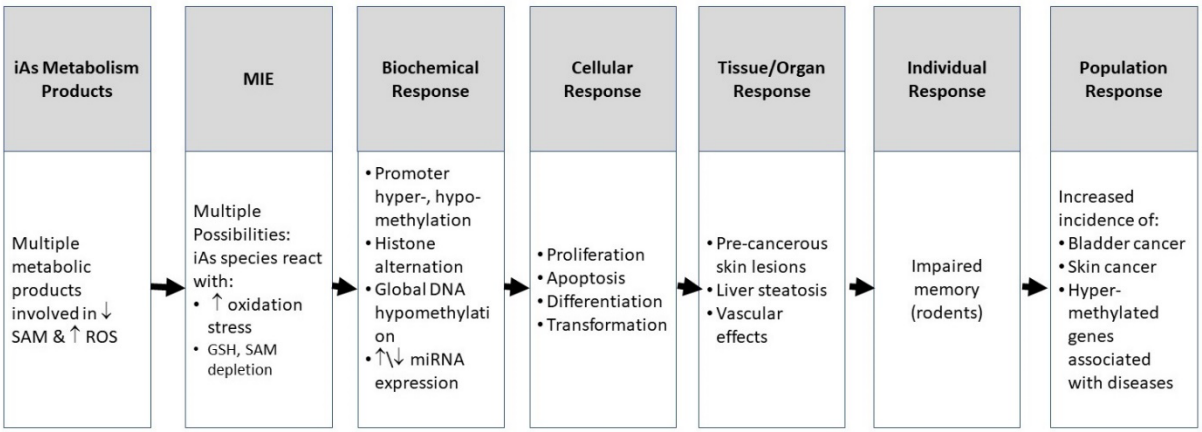


Figure A-3. Hypothesized mode of action for epigenetic mechanisms underlying associated health effects of inorganic arsenic exposures.

Abbreviations: Inorganic arsenic (iAs); molecular initiating events (MIEs); glutathione (GSH); S adenosylmethionine (SAM); miRNA = microRNA. See summary text and table for references; figure based on (Ankley et al., 2010).
Note: Figure shows a high -level summary of key events from the initial molecular interaction through a possible population -level response. The arrows link each key event (e.g., individual responses lead to population responses), but do not necessarily link each specific example response (e.g., impaired memory is not linked to skin cancer). Particularly note for this MOA that evidence at the individual level was only identified for effects related to impaired memory, even though population level responses indicate effects in other systems (e.g., bladder, skin). As the assessment development process moves forward, additional evidence may provide better understanding of the key events in this MOA and the connections between them.

A second major epigenetic response to inorganic arsenic exposure that the literature identifies is histone protein modifications. Histone proteins maintain the structure of chromatin and play an important role in gene transcription and repression. The most well–studied chemical modification of histones in response to inorganic arsenic exposure are changes in acetylation and methylation at concentrations as a low as 1 µM, but evidence also shows an association between inorganic arsenic and increased histone phosphorylation (Ren et al., 2011b).

An increasing body of evidence suggests that microRNA expression is altered in response to inorganic arsenic exposure (Kaul et al., 2014); (Li et al., 2012); (Rager et al., 2014); (Cao et al., 2011); (Marsit et al., 2006a) (Table A-4). MicroRNAs, which generally suppress the translation of mRNA into protein and enhance mRNA degradation, are both up- and downregulated (often in the same model system) after inorganic arsenic exposure.

While individual variation in responses have been widely reported after inorganic arsenic exposure, there are relatively few studies linking responses at the individual level to epigenetic changes. As discussed below, there are some data connecting health effects associated with inorganic arsenic exposures and epigenetic changes in population-based studies. One study on response at the individual level in animals did evaluate inorganic arsenic induced epigenetic changes in relation to cognitive function and found contextual memory deficits in rats exposed during gestation and early postnatal development (Martínez et al., 2011). (Martínez et al., 2011)

studied epigenetic modifications in Winstar rats resulting from arsenic exposure. In brief, Winstar rats were exposed to arsenic via drinking water at 3 and 36 ppm from gestation to four months of age. DNA methylation patterns in brain cells of the hippocampus and frontal cortex were then assessed. The results showed that arsenic altered methylation patterns in the cortex and hippocampus of exposed animals compared to controls starting at 1 month. The altered patterns of methylation in animals exposed to arsenic at 3 and 36 ppm correlated with progressive and dose-dependent aberrant memory effects (Martínez et al., 2011).

Based on available mechanistic and in vivo studies, a range of factors affecting individual variations in susceptibility may relate to epigenetic mechanisms underlying adverse health effects of inorganic arsenic exposures (Table A-4). These include dietary deficiencies, life stage susceptibility, gender, genetics, and smoking. Several studies have investigated the relationships between dietary sufficiency and epigenetic changes associated with inorganic arsenic exposure. Low folate status has been associated with the development of skin lesions in Bangladeshi adults (Pilsner et al., 2007), as well as HRAS promoter DNA hypomethylation, steatosis and microgranulomas in livers of mice exposed to inorganic arsenic (Okoji et al., 2002). While the proposed epigenetic MOAs suggest that dietary intake of methionine and folate intake would positively correlate with DNA methylation, conflicting evidence has been reported. Associations between increases in DNA methylation and inorganic arsenic exposure were only observed in individuals with adequate folate status (Pilsner et al., 2007). Moreover, (Lambrou et al., 2012) found that the exposure-response relationship between inorganic arsenic exposure and changes in DNA methylation in ALU retrotransport elements (thought to be involved in cancer and other diseases) varied depending on folate intake (Lambrou et al., 2012). Study subjects were elderly males from the Normative Aging Study whose arsenic exposures had been relatively low, (iAs concentrations ranging from 0.02 to 1.45 μg/g as measured in toenails). Evidence also suggests adverse effects related to folate supplementation and subsequent high fetal exposure to reactive iAs metabolites, where reduced fetal weights and altered fetal liver DNA methylation was observed after *in utero* exposure from mouse dams fed a high folate diet (Tsang et al., 2012).

In utero exposures to inorganic arsenic have been a major focus of efforts to identify susceptible life stages for epigenetic effects of iAs exposure. Studies in humans and rodents have detected DNA hypomethylation (Martínez et al., 2011); (Waalkes et al., 2004a) and numerous DNA methylation changes at specific loci (Tsang et al., 2012). Interestingly, the analysis of cord blood of inorganic arsenic exposed mothers revealed the upregulation of 12 miRNAs linked to cancer, diabetes, and immune response signaling pathways (Rager et al., 2014). Additionally, (Rojas et al., 2015) identified functional changes in the expression of genes highly enriched for binding sites of the early growth response (EGR) and CCC-TC binding factor (CTCF) transcription factors and health outcomes, including gestational age and head circumference, associated with CpG methylation.

The susceptible individual responses linked to genetic factors in different populations may shed light on population responses associated with the epigenetic mechanisms of inorganic arsenic-

induced adverse health outcomes. In addition, changes in DNA methylation patterns (hyper- or hypomethylation) have been identified in humans with skin and bladder cancers in arsenic endemic areas (arsenic exposure concentrations were 0.26 µg/g as measured in toenail samples of skin cancer patients and 50 µg/L iAs in drinking water in bladder cancer patients) (Chanda et al., 2006); (Marsit et al., 2006b). (Pilsner et al., 2009) found a relationship between global DNA hypomethylation at 121 µg/L in urine and the risk of inorganic arsenic-induced skin lesions. (Smeester et al., 2011) identified 182 genes whose promoter regions were consistently hypermethylated in a Mexican population with arsenicosis symptoms (skin lesions) in individuals exposed to a mean iAs of 110 µg/L. Notably, they also identified a network of 17 highly-methylated tumor suppressor and related genes (the "suppressome") and suggested that downregulation of these genes increased the risk of inorganic arsenic-associated cancer effects. These changes in DNA methylation patterns could serve as a molecular initiating event (MIE) in the overall iAs MOA.

Hypothesized Mode of Action (MOA): Cytotoxicity and Regenerative Proliferation

Relevant Health Effects: Bladder cancer, lung cancer, skin cancer

(Cohen et al., 2013) has proposed that the carcinogenic action of inorganic arsenic in the bladder is due to a mode of action (MOA) that includes cytotoxicity to urothelial cells followed by regenerative proliferation leading eventually to urothelial carcinoma. (Cohen et al., 2013) have further suggested that this MOA may also apply to lung and skin cancers. Prior to the molecular initiating events in this MOA, it is assumed that inorganic arsenic will be transformed into active metabolites. Under this MOA, exposure of sensitive tissue to the most toxic arsenic species, iAs(III) and MMA(III), and possibly thiolated species, results in the following sequence of events (Figure A-4):

- Reaction with sulfhydryl groups of specific proteins in the target tissue,
- Cytotoxicity caused by the reactive metabolites,
- Regenerative proliferation (including hyperplasia) in tissues (e.g., urothelium), and
- Development of tumors (Cohen et al., 2013)

(Cohen et al., 2013) and (Gentry et al., 2014) propose that, following ingestion and metabolism of relatively large amounts of inorganic arsenic (100-150 ppb in drinking water for humans), the molecular initiating event (MIE) under this MOA is the reaction of arsenic species with protein thiol groups in epithelial cells. The specific protein interactions responsible for the observed cytotoxicity and subsequent proliferation have not been identified, however (Cohen et al., 2013).

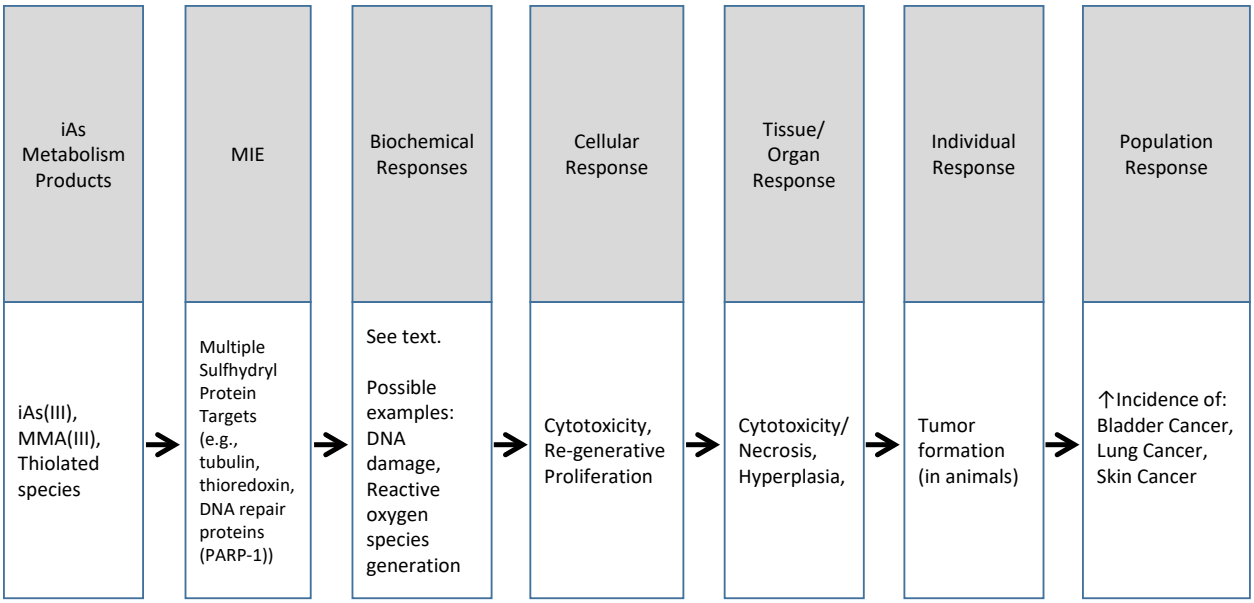


Figure A-4. Hypothesized mode of action for cytotoxicity and regenerative proliferation.

Abbreviations: Inorganic arsenic (iAs); Molecular Initiating Event (MIE); monomethylarsenous (MMA[III]); Poly [ADP-ribose] polymerase 1 (PARP-1). See Summary Text and Table for references; Figure based on (Ankley et al., 2010). Note: This Figure shows an overview of key events from the initial molecular interaction of arsenic species with sulfhydryl protein targets through a possible population level response. As the assessment development process moves forward additional evidence may provide better understanding of key events in the MOA and the level of evidence available to support connections between key events.

Cohen et al. (2013) also suggest that understanding underlying biochemical mechanisms (e.g., oxidative stress, epigenetic effects on DNA and histones), and the direct interaction of arsenic species with cellular signaling pathways is of limited relevance because the dose-response for the key cellular responses (cytotoxicity and proliferation) have been so well established.

The first proposed key cellular response that (Cohen et al., 2013) identify in this MOA is epithelial cell cytotoxicity. Evidence of cytotoxicity comes from a wide range of *in vitro* and in vivo studies. *In vitro*, the cytotoxicity of arsenic species (i.e., arsenite, MMA(III), DMA(III), and thiol derivatives) has been demonstrated in a number of primary and immortalized mammalian bladder cell lines (see Table A-2) (Styblo et al., 2000);(Drobna et al., 2003);(Cohen, 2002);(Sens et al., 2004);(Bredfeldt et al., 2006);(Eblin et al., 2008);(Suzuki et al., 2010). Cytotoxicity, as measured by LC_{50} or IC_{50}, varies greatly depending on the arsenic species being evaluated and the cell lines employed. *In vitro* acute cytotoxicity is greatest for the trivalent species (LC/IC_{50} values in the range of approximately 1–20 µM for iAs[III], MMA[III], DMA[III]) and lower for the pentavalent analogues (LC/IC_{50}s on the order of 30–1,500 µM). Acute cytotoxicity of trivalent arsenic appears similar in primary cell lines and immortalized (UROTSA) cells. Limited data on the thiol analogues such as DMMAT(V) suggest that its acute toxicity is similar to the trivalent arsenicals (LC_{50} = 1.4–5.5 µM in urothelial and bronchioepithelial cells, respectively).

Cytotoxicity and cellular necrosis have also been observed at the *organ or tissue* level in vivo in a number of studies where rats and mice were exposed to inorganic arsenic in diet and drinking water (Table in Section 10.1) however the doses used in these studies are much higher than human physiologically relevant doses (50–100 ppm in rodent studies versus ppb level exposures in humans)

Cohen et al. (2013) propose that the next key event in this MOA is increased cellular (regenerative) proliferation at the organ or tissue level, which was observed in several of the cytotoxicity studies just discussed (Table A-2). (Simeonova et al., 2000) observed urothelial hyperplasia and metaplasia in female C57BL/6 mice exposed to 0.01% sodium arsenite in drinking water for four weeks or longer. Hyperplasia was accompanied by a "cobblestone" appearance of the urothelium, but not by necrotic cytotoxicity. (Simeonova et al., 2000) subsequently observed urothelial hyperplasia and occasional squamous metaplasia in mice exposed to 50 and 100 μg/L iAs(III) for eight weeks. (Suzuki et al., 2008a), reported simple urothelial hyperplasia occurring roughly in parallel with increased cytotoxicity scores in rats and mice exposed to arsenite in food at 50–400 ppm or drinking water at 100 ppm for up to ten weeks. Subsequent studies with female rats confirmed a dose-dependent increase in cytotoxicity and urothelial hyperplasia following dietary exposures of 50 or 100 ppm for approximately 3–5 weeks (Suzuki et al., 2009); (Suzuki et al., 2010). (Yokohira et al., 2010) also observed both urothelial cytotoxicity and hyperplasia in C57BL/6 mice after as few as six days of exposure to 150 ppm arsenite in diet or four weeks exposure to 25 ppm arsenite in drinking water. Simultaneous occurrence of cytotoxicity and hyperplasia was confirmed by SEM observations in one mouse exposed to 150 ppm in food. The focus on low, non-cytotoxic concentrations in in vitro studies, and the use of transformed cell lines for evaluating indicators of proliferation (e.g., reduced doubling time) complicates further substantiating the sequential relationship of cytotoxicity and regenerative proliferation in this MOA (Sens et al., 2004); (Bredfeldt et al., 2006).

Inorganic arsenic has generally not been found to be carcinogenic in conventional rodent bioassays with adult animals. As discussed below, higher incidences of tumors in human populations with high exposures to inorganic arsenic suggest that this MOA is relevant for understanding adverse health outcomes in humans and emphasizes the importance of recent efforts to develop new rodent models of inorganic arsenic carcinogenicity (Cohen et al., 2013).

In contrast to data in adult animals, inorganic arsenic has been found to cause multisite tumors offspring of rodents after in utero exposures (Table A-2) (Waalkes et al., 2003); (Waalkes et al., 2004b); (Tokar et al., 2011). Dose-related increases in hyperplasia were also seen in several tissues, including the bladder, ovaries, and uterus of the females (0, 6, 12 or 24 ppm) in drinking water (Tokar et al., 2011).

Regarding population responses, Cohen et al. also suggest that the available epidemiological studies support the regenerative proliferative mechanism, in that increased arsenic-related cancer risk has only clearly been demonstrated in populations with exposure to relative high doses of

inorganic arsenic reviewed in (Cohen et al., 2013) (Table A-2). This would be consistent with a situation where increased cancer risk only occurred when internal concentrations of iAs(III) and/or other toxic metabolites reached levels associated with cytotoxicity, followed by regenerative proliferation and tumor development. However, increased population risks at higher concentrations are not observed in the analysis performed by (Cohen et al., 2013).

As(V) inhibition of Oxidative Phosphorylation (As(V) structural analog of phosphate)

Relevant Health Effects

In the cell, physicochemical similarities between As^{V} and phosphate result in substitution of As^{V} in a variety of chemical reactions in which phosphate would be the normal substrate. These reactions are commonly referred to as arsenolytic in that the substitution of As^{V} for phosphate forms a compound that is inherently unstable (Hughes et al., 2011). For example, As^{V}-containing esters that are readily formed as homologs of phosphate esters are quickly degraded. Although As^{V}-containing esters are inherently unstable, the formation of these compounds, typically at 100–200 µM in vitro, can disrupt normal phosphate metabolism in cells (Németi et al., 2010); (Gregus et al., 2009). However, this MOA is most likely to operate at higher As^{V} exposure levels due to normal physiological concentrations of phosphorous in human serum are approximately 3.4 mg/dL or 34,000 µg/L; (de Boer et al., 2009) (de Boer et al., 2009), therefore an individual exposed to 50 µg/L (50 ppb) arsenate is likely to have very little cellular uptake of arsenate due to competition with the relatively higher concentration (680-fold) of phosphate in serum

Hypothesized MOA: Endocrine Disruption

Relevant Health Effects: Developmental Neurotoxicity, Male Infertility, Prostate Cancer

Several adverse health effects following exposure to inorganic arsenic may result from events mediated by the endocrine system (Davey et al., 2008); (Goggin et al., 2012); (Gosse et al., 2014); (Prins, 2008) (Figure A-5). The molecular initiating event (MIE) in this MOA is a topic of ongoing research but based on literature reviewed for this summary may involve an interaction between inorganic arsenic and an element of the transcription complex for gene activation of nuclear hormone receptors. Specifically, inorganic arsenic may interact or modulate one of the following elements: (1) the hormone binding domain of the hormone receptor, (2) signaling pathways (e.g., mitogen activated protein kinases [MAPKs], extracellular signal-regulated kinases [ERK 1/2]) responsible for posttranslational modification of steroid hormone receptor proteins (e.g., coactivator phosphorylation), or (3) histone modifying proteins (i.e., acetylases, deacetylases, methylases) involved in receptor activation (Rosenblatt and Burnstein, 2009);(Barr et al., 2009);(Stoica et al., 2000). Notably, the first MIE option, interaction with the hormone binding domain, may be specific to estrogen receptor alpha (ERα), while the other possibilities may be more broadly applicable across both steroid receptors (e.g., glucocorticoid receptor [GR], progesterone receptor [PR], androgen receptor [AR], mineralocorticoids [MR]) and the larger class of nuclear

hormone receptors (e.g., thyroid hormone receptor [TR], retinoic acid receptor [RAR]) (Stoica et al., 2000);(Bodwell et al., 2006);(Davey et al., 2008).

Across receptor types, the literature indicates that the MIE is followed by a series of biochemical responses that can be broadly characterized as altering gene activation and subsequent cell signaling mediated by nuclear hormone receptors (Table A-3). In the case of ERα, inorganic arsenic may alter gene activation by inhibiting binding of the natural ligand, estradiol (E2), to the receptor (Stoica et al., 2000). Low levels of inorganic arsenic in vitro (1 nM) can then activate the receptor at levels approaching that of E2 (Stoica et al., 2000). Activation of ERα results in altered expression of genes regulated by the receptor (e.g., vitellogenin, pS2, PR), which is measurable at the mRNA and protein levels (Stoica et al., 2000). Importantly, inorganic arsenic activation of ERα gene transcription is likely mediated by the receptor since treatment with antiestrogen blocks gene transcription mediated by the receptor (Stoica et al., 2000).

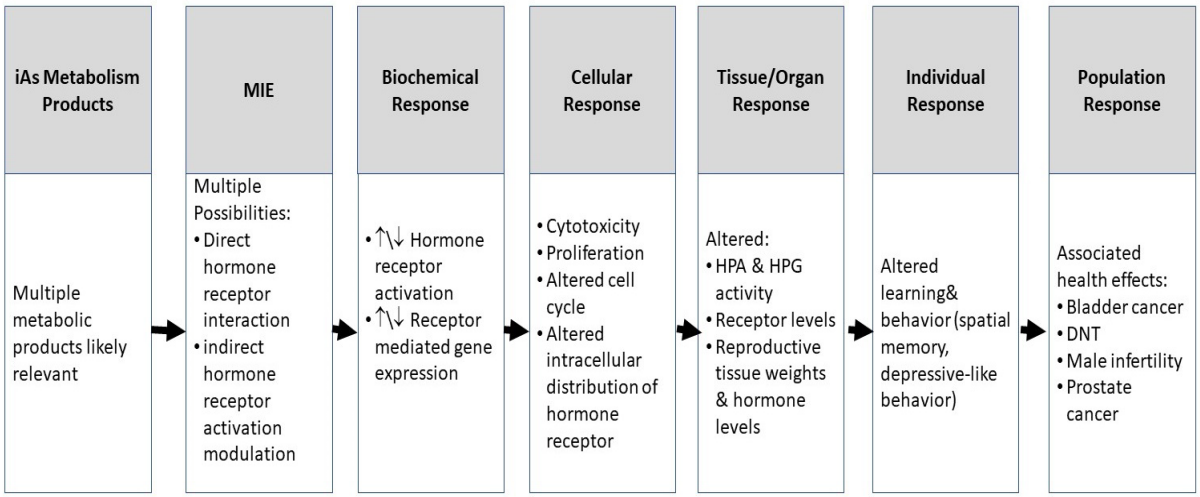


Figure A-5. Hypothesized mode of action for effects mediated by endocrine signaling.

Abbreviations: Inorganic arsenic (iAs); molecular initiating events (MIEs); hypothalamic-pituitary-adrenal (HPA); hypothalamic-pituitary-gondal (HPG); developmental neurotoxicity (DNT). See Summary Text and Table for references; Figure based on (Ankley et al., 2010). Note: Figure how a high-level summary of key events from the initial molecular interaction through a possible population level response. The arrows link each key event (e.g., individual responses lead to population responses), but do not necessarily link each specific example response (e.g., behavioral changes are not linked to male infertility). Of note for this MOA is that evidence at the individual level was only identified for effects related to developmental neurotoxicity, even though population level responses indicate effects in other systems (i.e., reproductive effects). As the assessment development process moves forward additional evidence may provide better understanding of the key events in this MOA and the connections between them.

While the above sequence of biochemical responses is supported by one group of investigators, others provide evidence that responses at the ERα receptor are similar to those of other nuclear hormone receptors (e.g., GR, PR, TR, RAR) (Stoica et al., 2000); (Davey et al., 2007). Under this second possible sequence of events, the MIE likely leads to alterations in

posttranslational modifications (e.g., phosphorylation) of coactivator proteins (e.g., TIF2, GRIP1) that are critical for transcriptional activity at response elements for each receptor (e.g., glucocorticoid receptor response elements [GREs]) (Barr et al., 2009);(Rosenblatt and Burnstein, 2009); these modifications may result in impaired interactions between coactivators (e.g., CARM1 and GRIP1) (Barr et al., 2009). Alternatively, the MIE may lead to alterations in histone modifications necessary for receptor-mediated gene activation (e.g., lower acetylation or methylation) (Barr et al., 2009). Ultimately, perturbations in the transcriptional complex impair receptor binding to response elements, leading to changes in receptor-mediated gene activation (Barr et al., 2009); (Rosenblatt and Burnstein, 2009). Changes in gene activation mediated by inorganic arsenic through this MOA may result in either activation or suppression of gene activity. Where low levels of inorganic arsenic (i.e., nanomolar range) may elevate hormone-mediated gene activation, higher (i.e., micromolar range), non-cytotoxic concentrations may suppress hormone-mediated gene activation (Davey et al., 2008); (Bodwell et al., 2004); (Bodwell et al., 2006). In addition to different outcomes resulting from low versus higher inorganic arsenic exposure levels, differences in levels of hormone receptors may underlie different responses across organ and tissue types (Bodwell et al., 2006).

Changes at the cellular level can ultimately lead to tissue or organ system responses that in this MOA include alterations in elements of the hypothalamic-pituitary-adrenal (HPA) axis (e.g., intracellular receptor distribution, protein glycosylation), the hypothalamic-pituitary-gonadal (HPG) axis (e.g., lower concentrations of gonadotropins and sex steroid hormones), testicular toxicity, impaired spermatogenesis, toxicity to the female reproductive system, and hormone-dependent tissue remodeling (i.e., morphogenesis) (Goggin et al., 2012);(Davey et al., 2008);(Sarkar et al., 2003);(Jana et al., 2006);(Chatterjee and Chatterji, 2010);(Chattopadhyay et al., 1999) (Table A-3. Data supporting alterations in the HPA axis are available from a developing animal model, suggesting that early life exposures to inorganic arsenic may have effects at the individual level, as discussed below (Goggin et al., 2012). Still other studies have suggested endocrine-mediated effects of inorganic arsenic exposure on male and female reproductive systems (e.g. decreased reproductive tissue weight, sperm count, infertility, altered activity of ovarian and testicular enzymes, and prostate cancer), which follows from alterations in elements of the HPG axis noted above at levels ranging from 53 µmol/L in mice (Pant et al., 2004) to up to 80 µg/mL in rats (Sarkar et al., 2003);(Jana et al., 2006);(Prins, 2008);(Chatterjee and Chatterji, 2010);(Chattopadhyay et al., 1999). Changes in morphogenesis were observed in an amphibian model of thyroid hormone (TH) activity that also has important implications for inorganic arsenic effects on TH during the perinatal period of human development (6 months of gestation through early postnatal development) (Goggin et al., 2012).

Little evidence was identified to link tissue or organ level responses to *individual responses* through this MOA; however, several studies suggest that alterations in GR transcription and subsequent changes in HPA axis activity, such as those outlined above, may lead to developmental

neurotoxicity (e.g., impaired stress response, depressive-like behaviors) following developmental inorganic arsenic exposure in mice (Goggin et al., 2012);(Martinez et al., 2008);(Martinez-Finley et al., 2011);(Martinez-Finley et al., 2009) (Table A-3).

No data were identified indicating the types of responses that might occur in *susceptible individuals* through this MOA. Given the role of steroid receptors in this MOA, differences in receptor or steroid levels across life stages or physiologic conditions may confer differences in response to inorganic arsenic exposures across individuals and provide insight on potentially susceptible individuals (Bodwell et al., 2006). The influence of receptor levels is particularly important in considering developmental inorganic arsenic exposures due to the critical role that TH, RAR and other nuclear hormone receptors play during development coupled with evidence of developmental neurotoxicity in animal models of inorganic arsenic exposure (Goggin et al., 2012); (Martinez et al., 2008); (Martinez-Finley et al., 2011); (Martinez-Finley et al., 2009); (Davey et al., 2008). Thus, pregnant women and developing children may be particularly susceptible to adverse outcomes from inorganic arsenic exposure.

Responses in susceptible individuals clearly influence responses observed at the *population level*. To that end, findings in rodents suggesting that endocrine effects may result in developmental neurotoxicity are concordant with findings in the epidemiology literature that show a correlation between early life exposure to inorganic arsenic and cognitive function (Wasserman et al., 2007). Other literature supports higher incidences of male infertility and prostate cancer in populations exposed to inorganic arsenic, although the connections between these observations and effects on the endocrine system are less clear.

As illustrated in the preceding sections inorganic arsenic is a toxicant that acts not through a single signal transduction pathway but rather, as the evidence demonstrates through multiple types of signal transduction pathways and modes of action that affect diverse biological functions from proliferation, apoptosis, autophagy, inflammation, inhibition of DNA repair enzymes, to epigenetic changes (see Tables A-1 through A-6).

A.1.2. DATA TABLES TO SUPPORT MOA ANALYSES

Table A-1. Preliminary data on effects mediated by oxidative stress

Relevant Health Effects: Cardiovascular Disease, Diabetes, Liver Disease, Lung Cancer, Bladder Cancer, Neurotoxicity, Non-Malignant Respiratory Disease, Pregnancy Outcomes, Renal Disease, Skin Cancer, and Skin Lesions

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
Molecular Initiating Events					
Reaction with O_2 (intermediate arsine species, e.g., dimethylarsine)	↑free radicals (e.g., Dimethylarsenic peroxyl radical [$(CH_3)_2AsOO$], superoxide anion)	Multiple (See Review Article)	Multiple (See Review Article)	Multiple (See Review Article)	Reviewed in (Flora, 2011)
Reaction with ferritin (Methylated-As)	Redox-active Fe release	Multiple (See Review Article)	Multiple (See Review Article)	Multiple (See Review Article)	Reviewed in (Flora, 2011)
Oxidation of As(III) to As(V)	H_2O_2 formation followed by Fenton reaction (hydroxyl radical formation)	Multiple (See Review Article)	Multiple (See Review Article)	Multiple (See Review Article)	Reviewed in (Flora, 2011); (Jomova and Valko, 2011)
Reactions with NADPH oxidase	↓ROS with NADPH inhibitor	Liver (Human)	Human immortalized liver cell line HL-7702	Diphenylene-iodonium chloride (DPI) (30 min pretreatment) + 5 µM arsenite (2 hr)	(Li et al., 2014); Reviewed in (Flora, 2011)
Reactions with mitochondrial respiratory chain	↓ROS with mitochondrial respiratory chain inhibitor	Liver (Human)	Human immortalized liver cell line HL-7702	Rotenone (30 min pretreatment) + 5 µM arsenite (2 hr)	(Li et al., 2014); Reviewed in (Flora, 2011)
Biochemical Responses					
Generation of reactive oxygen species	↓dichlorofluorescein diacetate (peroxides)	Skin (Human)	HaCaT transformed keratinocytes	0.5 µM trivalent arsenic (As[III]) (24 hr)	(Snow et al., 2005)
		Lung (Human)	WI38 human diploid lung fibroblast	0.5 µM trivalent arsenic (As[III]) (24 hr)	
	↑H_2O_2 ↑Superoxide	Lung (Rat)	Lung Epithelial Cells (LECs)	≤ 1 µM sodium arsenite (30 min)	(Li et al., 2011)
	↑Superoxide	Liver (Mouse)	Liver Sinusoidal Endothelial Cells (SECs)	2.5 – 5 µM arsenite (30 min)	(Straub et al., 2008)
Generation of reactive oxygen species (continued)	↑ 2',7'-dichlorofluorescein-diacetate (DCFH-DA)	Liver (Human)	Human immortalized liver cell line HL-7702	5 µM arsenite (2 hr)	(Li et al., 2014)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
	↑H_2O_2 *co-treatment with anti-oxidants prevents ↑	Liver (Rat)	Wistar Rats (Male, albino) (liver microsomes)	100 ppm sodium arsenite (30 d)	(Ramanathan et al., 2003)
		Kidney (Rat)	Wistar Rats (Male, albino) (kidney microsomes)	100 ppm sodium arsenite (30 d)	(Ramanathan et al., 2003)
	Dose dependent ↑CM-H_2DCFDA fluorescence (general ROS indicator) * co-treatment with anti-oxidants mitigates ↑ *latent ↑ with MMA(III) compared with As(III) (no ↑ at 10 min)	Urothelium (Human)	UROtsa cells	1–100 µM $NaAsO_2$ (10 min) 50 500 nM MMA(III) (30 min)	(Eblin et al., 2006)
	↑CM-H_2DCFDA * co-treatment with anti-oxidants mitigates ↑	Urothelium (Human)	UROtsa cells	10 µM $NaAsO_2$ (10 min) 500 nM MMA(III) (10 min)	(Eblin et al., 2008)
	Time-dependent ↑CM-H_2DCFDA fluorescence *significant ↑ only at 12 weeks	Urothelium (Human)	UROtsa cells	50 nM MMA(III) (4–12 weeks)	(Wnek et al., 2011)
Mitochondrial Activity Changes	↑colocalization of ROS & mitochondria staining	Liver (Human)	Human immortalized liver cell line HL-7702	5 µM arsenite (2 hr)	(Li et al., 2014)
Alteration in glutathione and other non-enzymatic antioxidant levels	↓GSH	Brain (Mouse)	Swiss Mice (Male albino)	0.5 or 1 As_2O_3 mg/kg (45 d)	(Rao and Avani, 2004)
		Brain (Rat)	Sprague Dawley Rats (Male)	0.05, 0.10, 0.30, 3.0 ppm Na_3AsO_4 (40 d)	(Chaudhuri et al., 1999)
		Lung (Rat)	Lung Epithelial Cells (LECs)	2 µM sodium arsenite (≤ 30 min)	(Li et al., 2011)
Alteration in glutathione and other non-enzymatic antioxidant levels (continued)	↓GSH ↓Ascorbic acid ↓α-tocopherol *co-treatment with anti-oxidants prevents ↓	Liver (Rat)	Wistar Rats (Male, albino) (liver microsomes)	100 ppm sodium arsenite (30 d)	(Ramanathan et al., 2003)
		Kidney (Rat)	Wistar Rats (Male, albino) (kidney microsomes)	100 ppm sodium arsenite (30 d)	(Ramanathan et al., 2003)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
	↑GSH	Pancreas (Rat)	Wistar Rats (Male)	1.7 mg/kg $NaAs^{+3}O_2$ (every 12 hr/ 90 d)	(Izquierdo-Vega et al., 2006)
		Pancreas (Rat)	INS-1(832/13) cells (Rat β-cells)	0.25 –0.5 µM arsenite (96 hr)	(Fu et al., 2010)
		Lung (Rat)	Lung Epithelial Cells (LECs)	2 µM sodium arsenite (2–8 hr)	(Li et al., 2011)
Depletion of micronutrients	↓ascorbate ↓ Fe(II)	Liver (Human)	Human immortalized liver cell line HL-7702	5 µM arsenite (12 hr)	(Li et al., 2014)
Enzyme Activity Changes	↓SOD dismutase ↓catalase	Brain (Mouse)	Swiss Mice (Male albino)	0.5 or 1 mg/kg As_2O_3 (45 d)	(Rao and Avani, 2004)
	↓SOD dismutase ↓catalase ↓glutathione reductase	Brain (Rat)	Sprague Dawley Rats (Male)	0.05, 0.10, 0.30, 3.0 ppm Na_3AsO_4 (40 d)	(Chaudhuri et al., 1999)
	↑DNA ligase	Lung (Human)	WI38 human diploid lung fibroblast	0.5 – 1 µM As(III) (24 to 120 hr)	Reviewed in (Snow et al., 2005)
	↓ DNA ligase			5 or 10 µM As(III) (24 to 120 hr)	
	Rac1-GTPase activation NADPH Oxidase activation (Nox2-based)	Liver (Mouse)	C57BL/6 Tac Mice (In-vivo and ex-vivo liver sinusoidal endothelial cells [SECs])	In vivo: 250 ppb sodium arsenite (5 wk) Ex vivo: 2.5 µM sodium arsenite (8 hr)	(Straub et al., 2008)
	↑ NADPH Oxidase (inferred) ↑Propyl hydroxylase (PHDs) (inactivates HIF-1α)	Liver (Human)	Human immortalized liver cell line HL-7702	5 µM arsenite (12 hr)	(Li et al., 2014)
Enzyme Activity Changes (continued)	↑haem oxygenase ↓Cytochrome P450 ↓Cytochrome b5 ↓NADPH-cyt P450 reductase *↑/↓ mitigated by antioxidants	Liver (Rat)	Wistar Rats (Male, albino) (liver microsomes)	100 ppm sodium arsenite (30 d)	(Ramanathan et al., 2003)
		Kidney (Rat)	Wistar Rats (Male, albino) (kidney microsomes)	100 ppm sodium arsenite (30 d)	(Ramanathan et al., 2003)
	↓thioredoxin reductase (TrxR)	Pancreas (Rat)	Wistar Rats (Male)	1.7 mg/kg $NaAs^{+3}O_2$ (every 12 hr/ 90 d)	(Izquierdo-Vega et al., 2006)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
	↓poly(ADP-ribose) polymerase-1 (PARP-1) *↑ activity if MMA(III) exposure is discontinued for 2 weeks prior to measurement in cells previously exposed for 4 or 8 weeks	Urothelium (Human)	UROtsa cells	50 nM MMA(II) (4–12 wks)	(Wnek et al., 2011)
Protein expression and/or level changes	Western Blot: ↑Base excision repair proteins (DNA polymerase β, DNA ligase I) ↓ Base excision repair proteins	Skin (Human)	Human Keratinocyte Cells (HaCaT)	0.1 – 1 µM As(III) (24 hr) 5–10 µM As(III) (24 hr)	Reviewed in (Snow et al., 2005)
	Western Blot: ↑Base excision repair proteins (DNA polymerase β, DNA ligase I) ↓ Base excision repair proteins	Lung (Human)	WI38 human diploid lung fibroblast	0.1 – 1 µM As(III) (24 hr) 5 –10 µM As(III) (24 hr)	Reviewed in (Snow et al., 2005)
	mRNA & Western Blot: ↑NRF1 ↑NRF2	Skin (Human)	Immortalized human keratinocyte cells (HaCaT)	>5 µM inorganic arsenite (As[III]) (6 hr)	(Zhao et al., 2012)
Protein expression and/or level changes (continued)	Western Blot: ↑Nrf2	Lung (Mouse)	Mice (unspecified strain; wild type and Nrf2-knockout)	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert background dust] (30 min/d /14 d)	(Zheng et al., 2012)
	Western Blot: ↑Cu/Zn SOD, thioredoxin *mitigated by antioxidants	Lung (Rat)	Lung Epithelial Cells (LECs)	2 µM sodium arsenite (16 wks)	(Li et al., 2011)
	Immunofluorescence: ↑PECAM-1	Liver (Mouse)	C57BL/6 Tac Mice (In-vivo and ex-vivo liver sinusoidal endothelial cells [SECs])	In vivo: 250 ppb sodium arsenite (5 wk) Ex vivo: 1–5 µM sodium arsenite (8 hr)	(Straub et al., 2008)
	Western Blot: ↑HIF-1α	Liver (Human)	Human immortalized liver cell line HL-7702	5 µM arsenite (12 hr)	(Li et al., 2014)
	Western Blot: ↑VEGF	Liver (Human)	Human immortalized liver cell line HL-7702	1–5 µM arsenite (12 hr)	(Li et al., 2014)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
	Western Blot: ↑Nrf nuclear fraction *↑ARE luciferase activity; ↑expression of downstream targets mRNA (e.g., Hmox1, NAD(P)H, catalase)	Pancreas (Rat)	INS-1(832/13) cells (Rat β-cells)	0.25 –0.5 µM arsenite (96 hr)	(Fu et al., 2010)
	Western Blot: ↑Hsp70 (stress protein)	Urothelium (Human)	UROtsa cells	1 µM $NaAsO_2$ (30 min) 10 µM $NaAsO_2$ (30–240 min)	(Eblin et al., 2006)
				50 nM – 5 µM MMA(III) (30 –240 min)	
	Western Blot: ↑metallothionein (stress protein)			1 µM $NaAsO_2$ (240 min) 10 µM $NaAsO_2$ (30–240 min)	
				50 nM – 5 µM MMA(III) (30 –240 min)	
Protein expression and/or level changes (continued)	mRNA & Western Blot: ↑PARP-1 *no effect on expression if MMA(III) exposure is discontinued for 2 weeks prior to measurement in cells previously exposed for 4 or 8 weeks	Urothelium (Human)	UROtsa cells	50 nM MMA(III) (4–12 wks)	(Wnek et al., 2011)
	Western blot: ↑ Cox-2 *levels normalize by 24 hr **co-treatment with SOD or melatonin block induction; no effect of catalase	Urothelium (Human)	UROtsa cells	1 µM sodium arsenite (4 hr); or 50 nM MMA(III) (4 hr)	(Eblin et al., 2008)
	mRNA: ↑ Cox-2 *levels normalize by 24 hr **co-treatment with catalase, SOD or melatonin block induction	Urothelium (Human)	UROtsa cells	1 µM sodium arsenite (4 hr); or 50 nM MMA(III) (4 hr)	(Eblin et al., 2008)
	Western blot: ↓Mn SOD *no change in Mn SOD with As(III) treatment; very little change in catalase with either As(III) or MMA(III) treatments	Urothelium (Human)	UROtsa cells	50 nM MMA(III) (1 to 24 hr)	(Eblin et al., 2008)
	Western blot: ↑Cu/Zn SOD *↓after 24 hr MMA(III) exposure	Urothelium (Human)	UROtsa cells	1 µM sodium arsenite (0.5 –24 hr) 50 nM MMA(III) (0.5 –4 hr)	(Eblin et al., 2008)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
Cell membrane disruption	↑Lipid peroxidation	Brain (Mouse)	Swiss Mice (male albino)	0.5 or 1 mg/kg As_2O_3 (45 d)	(Rao and Avani, 2004)
		Brain (Rat)	Sprague Dawley Rats (Male)	0.05, 0.10, 0.30, 3.0 ppm Na_3AsO_4 (40 d)	(Chaudhuri et al., 1999)
		Liver (Mouse)	BALB/c Mice (Male)	3.2 mg/L As(III)/As(V) (6 mos)	(Santra et al., 2000)
		Liver (Rat)	Wistar Rats (Male, albino) (liver microsomes)	100 ppm sodium arsenite (30 d)	(Ramanathan et al., 2003)
Cell membrane disruption (continued)	↑Lipid peroxidation (continued)	Kidney (Rat)	Wistar Rats (Male, albino) (Kidney microsomes)	100 ppm sodium arsenite (30 d)	(Ramanathan et al., 2003)
		Pancreas (Rat)	Wistar Rats (Male)	1.7 mg/kg $NaAs^{+3}O_2$ (every 12 hr/90 d)	(Izquierdo-Vega et al., 2006)
DNA, chromosomal damage	Oxidative DNA damage (↑anti-8-Oxo-dG staining)	Lung (Mouse)	Mice (unspecified strain; wild type and Nrf2-knockout)	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert background dust] (30 min/d /14 d)	(Zheng et al., 2012)
	Oxidative DNA damage (↑ 8-OHdG staining)	Blood (Human)	Human Population	10.88 to 19.05 µg/g Cr urinary arsenic (40–70 yrs)	(Pei et al., 2013)
	Oxidative DNA damage (↑anti-8-Oxo-dG levels measured by HPLC/ECD)	Urothelium (Human)	UROtsa cells	1 –10 µM $NaAsO_2$ (30 min)	(Eblin et al., 2006)
				50 nM MMA(III) (30) 50 nM – 5 µM MMA(III) (60 min)	
	↓anti-8-Oxo-dG levels measured by HPLC/ECD			1 – 10 µM $NaAsO_2$ (60 min)	
	↑DNA single-strand breaks (comet assay and flow cytometry)	Urothelium (Human)	UROtsa cells	50 nM MMA(II) (4–12 weeks)	(Wnek et al., 2011)
Gene expression changes	↑NRF2 & ARE dependent genes (HMOX-1, NQo1, GCLC, GCLM, SRX)	Skin (Human)	Immortalized human keratinocyte cells (HaCaT)	1.25–40 µM inorganic arsenite (As[III]) (6 hr)	(Zhao et al., 2012)
	↑Nrf2 targets (NQ01, γGCS, HO-1)	Lung (Mouse)	Mice (unspecified strain; wild type and Nrf2-knockout)	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert background dust] (30 min/d /14 d)	(Zheng et al., 2012)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
Gene expression changes (continued)	Altered gene expression related to: oxidative stress (↑HMOX1); protein folding (↓FKB5) Thioredoxin reductase (↑TXNRDI) Metallothinonine regulation (↑MT1E) DNA damage sensing (↓DDB2) Thioredoxin (↑TXN) Cell adhesion/growth (↑LGALS8) Immune response (↓THBD)	Urothelium (Human)	Human uroepithelial cells from kidney donor ureter segments	6 µM As(III) +MMA^{v}+DMA^{v} (24 hr); or 6 µM As(III) +MMA^{3+} +DMA^{3+} (24 hr)	(Clewell et al., 2011); (Yager et al., 2013)
	Alterations in genes related to: inflammatory signaling, epithelial-to-mesenchymal transition, cell cycle control, and apoptosis/survival signaling	Urothelium (Human)	Human uroepithelial cells from kidney donor ureter segments	0.06 µM inorganic arsenic and trivalent or pentavalent metabolites (24 hr)	(Clewell et al., 2011)
	↑adaptive gene response (delay apoptosis, preinflammatory)	Various	Various	≤ 0.01 µM various arsenic species (various exposure durations)	(Gentry et al., 2010) Review
	Altered gene expression related to: Oxidative stress, proteotoxicity, inflammation, and proliferative signaling, DNA repair, cell cycle, G2/M checkpoint control, and induction of apoptosis	Various	Various	0.1–10 µM various arsenic species (Various exposure durations)	(Gentry et al., 2010) Review
	Altered apoptotic gene expression	Various	Various	10 –100 µM various arsenic species (Various exposure durations)	(Gentry et al., 2010) Review
	760 Alternations in gene expression, generally related to: Oxidative stress (e.g., NQO1) Lipid metabolism (e.g., ALDH2) Inflammatory response (e.g., IL8, MAPK1)	Urothelium (Human)	UROtsa cells	1 µM MMA(III) (24 hr)	(Bailey et al., 2012)
Gene expression changes (continued)	176 alternations in gene expression, generally related to: Oxidative stress (e.g., TNF) Lipid metabolism (e.g., AKT3) Inflammatory response (e.g., IL8, IL6)	Urothelium (Human)	UROtsa cells	1 µM DMA(III) (24 hr)	(Bailey et al., 2012)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
	Genes in ERK 1/2 MAPK- & NF-KB signaling pathways	Urothelium (Human)	UROtsa cells	1 µM MMA(III) or DMA(III) (24 hr)	(Bailey et al., 2012)
Cell signaling changes. (Numerous; examples provided here—see review article for details)	Transcription Factors (e.g., Nrf2, HIF-1α, NF-κB)				Reviewed in (Flora, 2011)
	NF-κB (↑p-p65)	Lung (Mouse)	Mice (Unspecified strain; wild type and Nrf2-knockout)	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert background dust] (30 min/d /14 d)	(Zheng et al., 2012)
	Mitogen-activated protein kinases (MAPKs)				Reviewed in (Flora, 2011)
	Erk (Ras, Raf, MEK, ERK activation)	Lung (Rat)	Lung Epithelial Cells (LECs)	100 µM B[α]P (24hr) 2 µM sodium arsenite (16 wks)	(Li et al., 2011)
	Tyrosine phosphorylation				Reviewed in (Flora, 2011)
	↑p- Epidermal Growth Factor Receptor	Lung (Human)	Transformed human bronchial cells (BEAS)	500 µM sodium arsenite (20 min)	(Wu et al., 1999)
Cellular Responses					
Cytotoxicity/ viability, proliferation, apoptosis	↑cytotoxicity ↑apoptosis	Skin (Human)	Immortalized human keratinocyte cells (HaCaT)	>10 µM As(III) (24 hr)	(Zhao et al., 2012)
	↓cell viability *↑ mitigated by natural Nrf2-inducer	Lung (Human)	Human bronchial epithelium cells (16HBE14o)	≤ 1 µM As(III) (48 hr)	(Tao et al., 2013)
	↑ TUNEL labeling	Lung (Mouse)	Mice (Unspecified strain; wild type and Nrf2-knockout)	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert background dust] (30 min/d /14 d)	(Zheng et al., 2012)
Cytotoxicity/ viability, proliferation, apoptosis (continued)	↑proliferation	Lung (Rat)	Lung Epithelial Cells (LECs)	2 µM sodium arsenite (24 hr)	(Li et al., 2011)
	↑cell viability ↓cell viability *reduced Nrf2 expression sensitizes cells to viability change; activation of Nrf2 mitigates effects	Bladder (Human)	Human bladder urothelium cell line (UROtsa)	5–10 µM As(III) (24 hr) 20–80 µM As(III) (24 hr)	(Wang et al., 2007)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
	↓ cell viability *co-treatment with antioxidants other than catalase prevents ↓	Bladder (Human)	Human bladder urothelium cell line (UROtsa)	1 µM sodium arsenite, (24 hr)	(Eblin et al., 2008)
	No ↓ cell viability			50 nM MMA(III) (24 hr)	
Epithelial-mesenchymal transition	Colony formation, ↓epithelial protein markers ↑mesenchymal protein markers *mitigated by antioxidant treatment	Lung (Rat)	Lung Epithelial Cells (LECs)	100 µM B[α]P (24hr) 2 µM sodium arsenite (16 wks)	(Li et al., 2011)
Cell matrix changes	↓porosity	Liver (Mouse)	C57BL/6 Tac Mice (In-vivo and ex-vivo liver sinusoidal endothelial cells [SECs])	In vivo: 250 ppb sodium arsenite (5 wk) Ex vivo: 1–5 µM sodium arsenite (8 hr)	(Straub et al., 2008)
Functional Changes	↓insulin production ↓glucagon production	Pancreas (Rat)	Wistar Rats (Male)	1.7 mg/kg $NaAs^{+3}O_2$ (Every 12 hr/ 90 d)	(Izquierdo-Vega et al., 2006)
	↓insulin secretion in response to glucose ↑insulin secretion in response to potassium chloride	Pancreas (Rat)	INS-1(832/13) cells (Rat β-cells)	0.25–0.5 µM arsenite (96 hr)	(Fu et al., 2010)
Malignant transformation	↑multinucleated cells, morphological changes (confocal microscopy) tumor formation in in vivo xenografts	Urothelium (Human)	UROtsa cells	0.05 µM MMA(III) (24–52 wks)	(Bredfeldt et al., 2006)
Tissue/ Organ Responses					
Tissue remodeling	↑ Alveolar septa thickening, collagen deposition, fibroblast proliferation, pneumocyte hyperplasia;	Lung (Mouse)	Mice (Unspecified strain; wild type and Nrf2-knockout)	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert background dust] (30 min/d /14 d)	(Zheng et al., 2012)
Inflammatory response	↑ inflammatory cells in BAL fluid ↑TNF-α, IL-6 in BAL fluid ↑Th2 cytokines (IL-3, IL-4) ↑chemokines (TGF-β, MCP-1) *↑ mitigated by natural Nrf2-inducer	Lung (Mouse)	Mice (Unspecified strain; wild type and Nrf2-knockout)	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert background dust] (30 min/d /14 d)	(Zheng et al., 2012)
	↑ TNF-α, IL-1β, IFNγ	Placenta (Human)	Human Population	>60 µg/L urinary arsenic at gestational week 30	(Ahmed et al., 2011)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
Vascular remodeling	Sinusoidal capillarization ↓nutrient/ waste exchange	Liver (Mouse)	C57BL/6 Tac Mice (In vivo and ex vivo liver sinusoidal endothelial cells [SECs])	In vivo: 250 ppb sodium arsenite (5 wk) Ex vivo: 8 hr	(Straub et al., 2008)
Endocrine signaling changes	↑fasting serum glucose ↑blood insulin	Pancreas (Rat)	Wistar Rats (Male)	1.7 mg/kg $NaAs^{+3}O_2$ (Every 12 hr/90 d)	(Izquierdo-Vega et al., 2006)
Individual Responses					
Diabetes (Inferred from insulin resistance)	Insulin resistance	Blood (Rat)	Wistar Rats (Male)	1.7 mg/kg $NaAs^{+3}O_2$ (every 12 hr/ 90 d)	(Izquierdo-Vega et al., 2006)
Liver disease	Hepatic fibrosis	Liver (Mouse)	BALB/c Mice (Male)	3.2 mg/L (15 mos)	(Santra et al., 2000) Reviewed in (Flora, 2011)
Non-malignant respiratory disease	Allergic lung inflammation	Lung (Mouse)	Mice (Unspecified strain; wild type and Nrf2-knockout)	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert background dust] (30 min/d /14 d)	(Zheng et al., 2012)
Susceptible individual response					
KEAP1 and/or NRF2 mutations	↑ NRF2 activity in skin cancer patients	Skin	Human population	Not applicable	(Kim et al., 2010) cited in (Zhao et al., 2012)
NADPH oxidase p22 subunit polymorphisms	↑hypertension risk in individuals with polymorphisms & high inorganic arsenic exposure	Cardio-vascular system	Human population	0.7–0.93 mg/L median inorganic arsenic in well water (>6 mos)	(Hsueh et al., 2005); Cited in (Straub et al., 2008)
Diabetics	↓thioredoxin reductase (TrxR)	Pancreas (Rat)	Wistar Rats (Male)	1.7 mg/kg $NaAs^{+3}O_2$ (Every 12 hr/ 90 d)	(Izquierdo-Vega et al., 2006); (Schulze et al., 2004)
Alcohol	Ethanol may augment oxidative stress and induction of angiogenic factors that would promote tumor growth	Cardiovascular system	Human microvascular endothelial (HMVEC) cells	1–5 µM arsenite in presence or absence of 0.1% EtOH. 24-hr experiments	(Klei and Barchowsky, 2008)
Population Response[a]					
Elevated oxidative stress	↑superoxide in plasma (Chemiluminescence method) ↓Plasma antioxidants	Plasma (Human)	Human Population (Taiwan)	9.60 µg/L Average arsenic blood levels (Average age: 64 yrs)	(Wu et al., 2001)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
Elevated oxidative stress	↑serum lipid peroxides ↓non-protein sulfhydryl levels in whole blood	Blood (Human)	Human population (Inner Mongolia, China,)	0.41 mg/L Average arsenic blood levels (Average: 18 yrs)	(Pi et al., 2002)
Cardiovascular disease	Peripheral vascular disease, ischemic heart disease, acute myocardial infarction, atherosclerosis, hypertension	Cardiovascular system	Human population	Varies	Cited by (Straub et al., 2008) Reviewed in (Flora, 2011)
Bladder cancer	Elevated incidence of bladder cancer in populations exposed to relatively high inorganic arsenic concentrations (>100 µg/L in drinking water)	Bladder	Human population	Varies but generally >100 µg/L in drinking water	Reviewed in (Cohen et al., 2013)
Diabetes	Multiple measures (e.g., insulin resistance)	Endocrine system	Human population	Various	(Maull et al., 2012); cited in (Fu et al., 2010)
Liver Cancer	↑serum Epidermal Growth Factor Receptor in liver cancer patients	Serum	Human Case Controls	Average 0.5–0.6 mg/L inorganic arsenic in drinking water	(Sung et al., 2012)
Liver disease	Portal hypertension, noncirrhotic liver fibrosis	Liver	Human population	Various	Cited in (Straub et al., 2008)
	Hepatic fibrosis, portal hypertension	Liver	Human population	Various	(Santra et al., 1999); Reviewed in (Flora, 2011)
Lung Cancer	Inferred from EGFR activation in BEAS cells and ↑EGFR in serum of liver cancer patients	Lung	Human population	Various	(Wu et al., 1999); (Sung et al., 2012)
Neurotoxicity	Peripheral neuropathy	Nervous system	Human population	Various	Cited by (Rao and Avani, 2004)
Non-malignant respiratory disease	Allergic lung inflammation	Lung	Human population	Various	Cited in (Zheng et al., 2012)
Pregnancy outcomes	preeclampsia, pre-term birth, chorioamnionitis, brain white matter damage, chronic lung disease in preterm infants	Placenta (Human)	Human population	Various	Cited in (Ahmed et al., 2011)
Renal disease	Urinary cancer Renal insufficiency, necrosis, failure	Kidney	Human population	Various	Reviewed in (Flora, 2011)

Key events	Observations	Organ system	Test system	Dose (exposure duration)	References
Skin Disease (Bowmen's Disease, cancer)	↑oxidative DNA adducts. (8-OHdG) ↑skin lesions	Skin	Human population	Various	(Pei et al., 2013) Reviewed in (Yu et al., 2006)

[a]Note: Associations between disease in populations exposed to inorganic arsenic and oxidative stress relies primarily on observational population studies combined with indicators of oxidative stress in in vitro and/or in vivo studies in cell or tissue types relevant to the disease (e.g., cardiomyocytes for cardiovascular disease). Data directly linking inorganic arsenic exposure to disease through an oxidative stress MOA were not identified at the population level, although biomarkers of oxidative stress in populations exposed to inorganic arsenic have been identified.

Table A-2. Preliminary data on effects mediated by cytotoxicity and regenerative proliferation

1 *Relevant Health Effects: Bladder, lung, and skin cancer*

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Molecular Initiating events					
Reactions with GSH and other non-protein thiols	Glutathione, cysteine, lipoic acid conjugates	Many thing to the effect of "several loines of evidence support several different modes of action	Humans, rodents, in vitro	Environmentally relevant and higher exposures	(Cohen et al., 2013)
Reaction with thiols/ dithiols in specific proteins	Inorganic arsenic binding with tubulin, keratin, ER-α and related receptors, PARP-1, thio-redoxin reductase, As3mt, KEAP-1, many studies of zinc finger proteins, peptides; IκB kinase; EGFR, Shc; tyrosine phosphatases, ubiquitination enzymes; XPA, XPD (NER enzymes)	Not applicable	In vitro binding of As(III) to synthetic peptides	Kds =~1–30 µg/L (↓Kd with ↑cysteine residues)	(Kitchin and Wallace, 2005); (Kitchin and Wallace, 2008), (Qin et al., 2008)
	Reduced PARP activity, restored by co-incubation with Zn	Urothelium (Human)	UROtsa cells	50 nM MMA(III) (12–52 wks)	(Wnek et al., 2011); (Wnek et al., 2009)
Biochemical Responses					

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
See summary text					(Cohen et al., 2013)
Cellular Responses					
Cytotoxicity/ viability	24-hour viability (mitochondrial dehydrogenase assay)	Urothelium (Human)	UROTSA, other cell lines	Arsenite IC_{50} for UROTSA = 17.8 µM, 3.2 µM for bronchial cells, 10 µM for rat hepatocytes, >20 µM for human hepatocytes, keratinocytes (24 hr)	(Styblo et al., 2000)
Cytotoxicity/ viability (continued)	24-hour viability (mitochondrial dehydrogenase assay)	Multiple	Primary human, rat hepatocytes, 13 mammalian cell lines	IC_{50}s (24 hrs): As(III) = 1–100 µM; MMA(III): 0.4 – 5.5 µM; DMA(III): 0.4 – >20 µM; most sensitive cell line: MB4 (human leukemia-derived)	(Styblo et al., 2000)
	Cell viability (light microscopy); 95% mortality at low exposure, >99% mortality at two highest exposures	Urothelium (Human)	UROtsa cells	1 µM As(III) (30 – 48 d) 4, 8 µM As(III) (30 d)	(Sens et al., 2004)
	Viability (MTT) assay	Urothelium (Human)	UROtsa cells	IC_{50} ~5 µM MMA(III) (24–72 hr) "threshold" for viability & morphology changes: ~2 µM	(Bredfeldt et al., 2006)
	Viability ↓ 42% (Trypan blue assay) *reduction, partially abolished by ROS scavengers	Urothelium (Human)	UROtsa cells	1 µM As(III) (24 hr)	(Eblin et al., 2008)
	Viability ↓ (Trypan blue assay) *reduction, partially abolished by NADPH oxidase inhibitor, but other antioxidants	Urinary bladder epithelium (Rat)	MYP3 rat cell line	1 µM As(III) (3 d)	(Suzuki et al., 2009)
	Viability ↓ (Trypan blue assay)	Urinary bladder epithelium (Rat)	MYP3 rat cell line	LC50: 0.75 µM As(III) (3 d)	(Suzuki et al., 2010)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
		Ureter epithelium (Human)	1T1 human cell line	8.3 µM As(III) (3 d)	
Proliferation	Reduced doubling time (43.1 hr to 22.1 hr)	Urothelium (Human)	UROtsa cells	1 µM As(III) (>60 d)	(Sens et al., 2004)
	Reduced doubling time (42 hr to 27 hr)	Urothelium (Human)	UROtsa cells	50 nM MMA(III) (12 wks)	(Bredfeldt et al., 2006)
	Reduced doubling time (42 hr to 21 hr)	Urothelium (Human)	UROtsa cells	50 nM MMA(III) (52 wks)	(Bredfeldt et al., 2006)
	↑thymidine uptake ↑S-phase cells ↓G_0/G_1 cells	Urothelium (Human)	UROtsa cells	2 or 4 µM sodium arsenite (48–72 hr) 2 or 4 µM sodium arsenite (24 hr)	(Simeonova et al., 2000)
Malignant transformation	Colony formation in soft agar, tumor formation after hetero transplantation	Urothelium (Human)	UROtsa cells	1 µM As(III) (60 d, followed by repeated passages in As-free medium)	(Sens et al., 2004)
	Colony formation in soft agar	Urothelium (Human)	UROtsa cells	50 nM MMA(III) (24 or 52 wks)	(Bredfeldt et al., 2006)
	Differentiation to squamous epithelium with poorly defined cell membranes, multinucleate cells, tumor formation after hetero-transplantation in SCID mice; ↑proliferative biomarker (Ki-67) in tumors	Urothelium (Human)	UROtsa cells	50 nM MMA(III) (52 wks)	(Bredfeldt et al., 2006)
Tissue/ Organ Responses					
Tissue Cytotoxicity/ Necrosis	Mild-moderate urothelial cytotoxicity (observed by scanning electron microscopy [SEM])	Urothelium (Rat; Mouse)	F344 rats, C57BL/6 mice	100 µg/L As(III) in drinking water (2 wks) ; or 50–400 µg/g in diet (2–10 wks)	(Suzuki et al., 2008b)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
	Urothelial cytotoxicity, (observed by SEM) *Cytotoxicity reduced by NADPH oxidase inhibitor, apocyanin (250 mg/L)	Urothelium (Rat)	F344 rats (Female)	100 ppm As(III) in diet (20 d)	(Suzuki et al., 2009)
Tissue Cytotoxicity/ Necrosis (continued)	Urothelial cytotoxicity, necrosis (observed by SEM)	Urothelium (Rat)	F344 rats (Female)	Dose-response ~10–50 ppm As(III) in diet (5 wks) (NOEL: 1–10 ppm; significant at ≤ 50 ppm)	(Suzuki et al., 2010)
	Urothelial cytotoxicity, necrosis (observed by SEM) *cytotoxicity in KO compared to WT in same treatment groups	Urothelium (Mouse)	Wild Type (WT) and arsenic methyl-transferase (As3mt) KO mice (Female)	100 ppm As(III) in diet (6 d), followed by 50 ppm in drinking water (3 d)	(Yokohira et al., 2010)
	Urothelial cytotoxicity, necrosis (observed by SEM) *cytotoxicity in KO compared to WT in same treatment groups	Urothelium (Mouse)	WT and As3mt KO mice (Female)	10–25 ppm As(III) in drinking water (4 wks)	(Yokohira et al., 2011)
	Mild-moderate urothelial cytotoxicity (observed by SEM) *severity increased over time	Urothelium (Rat)	F344 rats (Female)	100 ppm As(III) in drinking water (6 hr–14 d)	(Arnold et al., 2014)
		Urothelium (Mouse)	C57BL/6 WT and As3mt KO mice (Female)	25 ppm As(III) in drinking water (6 hr – 14 d)	
Tissue regeneration/ Hyperplasia	Mild-moderate urothelial hyperplasia (male and female rats, male mice)	Urothelium (Rat; Mouse)	F344 rats; C57BL/6 mice	100 µg/L As(III) in water; 50–400 µg/g in diet (2–10 wks)	(Suzuki et al., 2008b)
	Urothelial hyperplasia *No effect of co-exposure to NADPH oxidase inhibitor	Urothelium (Rat)	F344 rats (Female)	100 ppm As(III) in diet (20 d)	(Suzuki et al., 2009)
	Urothelial hyperplasia	Urothelium (Rat)	F344 rats (Female)	~10–100 ppm As(III) in diet (5 wks) (NOEL: 1–10 ppm; significant at ≤ 50 ppm)	(Suzuki et al., 2010)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Tissue regeneration/ Hyperplasia (continued)	Mild-moderate hyperplasia, *greater severity in KO strain, but NOEL of 1 ppm in both strains;	Urothelium (Mouse)	WT and As3mt KO mice (Female)	50 ppm As(III) in drinking water (6 days); or 10–25 ppm As(III) in drinking water (4 wks)	(Yokohira et al., 2011)
	Mild to moderate bladder hyperplasia (cancer bioassay)	Urinary bladder (Rat)	F344 rats	40 or 100 ppm DMA(V) in feed (2 yrs)	(Arnold et al., 2006)
	Urinary bladder hyperplasia *observed at all exposure levels in males; only observed in lowest exposure group in females	Urinary bladder (Mouse)	CD-1 mice	6, 12, 24 ppm sodium arsenite (2 wks prior to parental mating through 2 yrs in adulthood)	(Tokar et al., 2011)
	Urothelial hyperplasia *increased severity & incidence over time	Bladder epithelium (Rat)	F344 rats (Female)	100 ppm As(III) in drinking water (24 hr–14 d)	(Arnold et al., 2014)
Hyperplasia and Metaplasia	Urothelial hyperplasia, occasional metaplasia	Urinary Bladder (Mouse)	C57/BL-6 mice (Female)	0.01% sodium arsenite in drinking water (4 wks)	(Simeonova et al., 2000)
Individual Responses					
Tumor development (animals)	Urothelial cell papillomas *statistically significant positive trend if combine male & female Urothelial cell carcinomas *statistically significant positive trend in females if male & female data combined (low incidence in males precludes statistical analysis	Urinary Bladder (Rat)	F344 rats	2–100 ppm DMA(V) in feed (2 yrs)	(Arnold et al., 2006)
	No increase in tumor incidence	Urinary Bladder (Mouse)	B6C3F1 mice	8, 40, 200, or 500 ppm DMA(V) in feed (2 yrs)	(Arnold et al., 2006)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Tumor development (animals) (continued)	dose-related ↑ in: hepatocellular carcinomas, adrenal tumors (male offspring); lung carcinomas, ovarian tumors, proliferative lesions of oviduct and uterus (female offspring)	Multiple Tissues (Mouse)	C3H mice	42.5, 85 ppm sodium arsenite in drinking water (gestation days 8–18)	(Waalkes et al., 2003); (Waalkes et al., 2004b)
	Increased tumor incidence of liver, lung, gall bladder, adrenal gland kidney (male offspring); Liver, lung, ovary, uterus (female offspring)	Multiple Tissues (Mouse)	CD-1 mice	6, 12, 24 ppm sodium arsenite (2 wks prior parental to mating through 2 yrs in adulthood)	(Tokar et al., 2011)
Susceptible Individuals					
Reduced As methylation capacity	Subjects with lower secondary methylation indices had higher risk of skin and bladder cancer	Skin Urinary bladder (Human)	Human Population	Cumulative inorganic arsenic intake 0–20 mg/L-yr	(Chen et al., 2003a); (Chen et al., 2003b)
Cytotoxicity, regenerative proliferation associated with urinary calculi	Observations of mild cytotoxicity, regenerative proliferation after exposure to calculi-inducing substances	Urinary bladder (Human)	Animals and human population	Drugs (humans) and wax implants (animals)	(Cohen, 2002)
UV-exposure	↑UV-induced DNA strand breaks	Skin (Human)	HaCat cells	1 µM sodium arsenite (24 hr)	(Qin et al., 2008)
	↓ UV-induced DNA repair enzyme activity			2 µM sodium arsenite (24 hr)	
Human Population Responses					

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Inorganic arsenic-associated cancer risk (bladder, lung, skin)	Elevated risks of bladder, lung, and skin cancer in chronically inorganic arsenic-exposed populations (multiple epidemiological studies); primarily limited to populations with water As levels >100 µg/L; limited data suggest urinary inorganic arsenic at levels found to be cytotoxic in rodents are associated with elevated risks. Liver, prostate cancer risk associated with inorganic arsenic (smaller number of studies)	Multiple tissues (Human)	Humans	Wide range of exposure levels and durations	Reviewed in: (Cohen et al., 2013), (Gibb et al., 2011), (Schoen et al., 2004), (NRC, 1999)

[a]Exposure duration abbreviations: minutes (min), hours (hr), days (d), weeks (wks), years (yrs)

Table A-3. Preliminary data on effects mediated by endocrine

1 *Relevant Health Effects: Developmental Neurotoxicity, Male Infertility, Prostate Cancer*

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Molecular Initiating Events					
Interaction with hormone binding domain in hormone receptors	↑reporter activity of ERα hormone binding domain *inhibited by antiestrogen	Kidney (Monkey)	COS-1 cells	1 µM sodium arsenite (24 hr)	(Stoica et al., 2000); (Rosenblatt and Burnstein, 2009); (Barr et al., 2009)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Modulate signaling pathways (e.g., mitogen activated protein kinases [MAPKs, extracellular signal-regulated kinases [ERK1/2]) responsible for posttranslational modification of coactivators or steroid hormone receptors	Hypothesis	Not applicable (N/A)	N/A	N/A	(Rosenblatt and Burnstein, 2009); (Barr et al., 2009)
Modulate histone modifying proteins (e.g., acetylases, methylases) responsible for posttranslational modification of coactivators or steroid hormone receptors	Hypothesis	N/A	N/A	N/A	(Rosenblatt and Burnstein, 2009); (Barr et al., 2009)
Biochemical Responses					
Alterations in Nuclear Hormone Receptor Mediated Gene Activation					
Androgen Receptor (AR)					
↓AR amino and carboxyl (N-C) termini interaction	↓luciferase activity in mammalian two-hybrid assay	Prostate (Human)	PC3 cells (human prostate cancer cells)	5 µM arsenic trioxide (ATO) (24 hr)	(Rosenblatt and Burnstein, 2009)
↓AR coactivator-stimulated N-C interaction	↓luciferase activity in mammalian two-hybrid assay	Prostate (Human)	PC3 cells (human prostate cancer cells)	5 µM ATO (24 hr)	(Rosenblatt and Burnstein, 2009)
↓AR coactivator recruitment to chromatin	↓ immuno-precipitation of TIF2 at Prostate-Specific Antigen (PSA) promoter	Prostate (Human)	LNCaP cells (human prostate cancer cells)	5 µM ATO (24 hr)	(Rosenblatt and Burnstein, 2009)
↓AR recruitment to chromatin	↓chromatin immuno-precipitation of AR at PSA promoter	Prostate (Human)	LNCaP cells	5 µM ATO (24 hr)	(Rosenblatt and Burnstein, 2009)
↓AR-mediated gene activation	↓ androgen response element luciferase activity (ARE or PSA)	Prostate (Human)	PC3, LNCaP, or LAPC4 cells (human prostate cancer cells)	1–5 µM ATO (48 hr)	(Rosenblatt and Burnstein, 2009)
	↓androgen response element luciferase activity	Testes (Mice)	TM4 mouse Sertoli cells	2 µM ATO (48 hr)	(Rosenblatt and Burnstein, 2009)
	↓PSA mRNA	Prostate (Human)	LNCaP cells	2 µM ATO (48 hr)	(Rosenblatt and Burnstein, 2009)
Estrogen Receptor (ER)					

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Inhibition of estradiol binding to ERα	↓[3H] estradiol binding *not seen in work by Chow et al., Chow et al. (2004) using ERα competitive screening kit	Breast (Human)	Human breast cancer MCF-7 cells	Ki: 0.5nM sodium arsenite (18 hr)	(Stoica et al., 2000)
	No ↓[3H] estradiol binding	Breast (Human)	Biochemical assay (screening kit)	100–200 nM ATO (not specified)	(Chow et al., 2004)
↑ERα activation	↑estrogen response element reporter construct activity in ERα	Kidney (Monkey)	COS-1 cells	1 nm–10 µM sodium arsenite (24 hr)	(Stoica et al., 2000)
Altered ER-mediated gene activation	↓vitellogenin expression (mRNA)	Liver (Chicken)	Chick Embryo	10 – 50 µmol/kg As(III) (4 hr) 10 µmol/kg E2 (3 hr)	(Davey et al., 2007)
	↓Estrogen Response Element expression (luciferase expression or mRNA)	Breast (Human)	Human breast cancer MCF-7 cells	2.5 µM As(III) (EC50) (24 hr)	(Davey et al., 2007)
	↓GREB1 basal (mRNA)	Breast (Human)	Human breast cancer MCF-7 cells	5 µM As(III) (EC50) (24 hr)	(Davey et al., 2007)
	↓GREB1-E2 induced (mRNA)	Breast (Human)	Human breast cancer MCF-7 cells	5 µM As(III) (EC50) (24 hr)	(Davey et al., 2007)
	↓ERα basal (mRNA)	Breast (Human)	Human breast cancer MCF-7 cells	5 µM As(III) (EC50) (24 hr)	(Davey et al., 2007); (Stoica et al., 2000)
Altered ER-mediated gene activation (continued)	↓ERα basal (mRNA	Breast (Human)	Human breast cancer MCF-7 cells	2 µM ATO (24 or 48 hr)	(Chow et al., 2004)
	↓ERα hormone induced (mRNA) *synergistic ↓ with E2	Breast (Human)	Human breast cancer MCF-7 cells	2 µM ATO + 10 nM estradiol (24 or 48 hr)	(Chow et al., 2004)
	↓Estrogen Response Element expression (luciferase expression)	Breast (Human)	Human breast cancer MCF-7 cells	2 µM ATO (24 or 48 hr) 2 µM ATO + 10 nM estradiol (24 or 48 hr)	(Chow et al., 2004)
	↓c-myc protein	Breast (Human)	Human breast cancer MCF-7 cells	2 µM ATO (48 hr)	(Chow et al., 2004)
	↓c-myc protein induced by E2			2 µM ATO + 10 nM estradiol (48 hr)	
	↑pS2 (mRNA) *↑blocked by antiestrogen	Breast (Human)	Human breast cancer MCF-7 cells	1µM sodium arsenite (24 hr)	(Stoica et al., 2000)
↓ER-mediated protein levels	↓ERα protein	Breast (Human)	Human breast cancer MCF-7 cells	0.1, 1, or 5 µM sodium arsenite (24 hr)	(Stoica et al., 2000)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
	↓ERα protein	Breast (Human)	Human breast cancer MCF-7 cells	2 µM ATO (48 hr)	(Chow et al., 2004)
	↓ERα hormone induced protein *synergistic ↓ with E2			2 µM ATO + 10 nM 17β-estradiol (48 hr)	
	↑progesterone receptor protein *↑blocked by antiestrogen	Breast (Human)	Human breast cancer MCF-7 cells	1 µM sodium arsenite (24 hr)	(Stoica et al., 2000)
	↓Vascular Endothelial Growth Factor protein (mRNA and protein)	Uterus (Rat)	Sprague-Dawley rats (Female)	4 µg/mL sodium arsenite (28 d)	(Chatterjee and Chatterji, 2010)
Glucocorticoid Receptor (GR)					
Altered histone post-translational co-activator protein activity at GR-regulated promoter	↓protein methyltransferase (CARM1) / coactivator (GRIP1) interaction	Tumor (Mouse)	1470.2 cells (mouse adenocarcinoma derived)	8 µM sodium arsenite + 5 nM dexamethasone (Dex) (30 min)	(Barr et al., 2009)
Altered histone post-translational modifications at GR-regulated promoter	↓ acetylation (H3K18ac) ↓methylation (H3R17me)	Tumor (Mouse)	1470.2 cells (mouse adenocarcinoma derived)	8 µM sodium arsenite + 5 nM Dex (15 min)	(Barr et al., 2009)
↓chromatin remodeling at GR regulated promoter	↓A Sac1 endonuclease cleavage site access	Tumor (Mouse)	1470.2 cells (mouse adenocarcinoma derived)	8 µM sodium arsenite + 5 nM Dex (30 and 60 min)	(Barr et al., 2009)
↓GR binding to glucocorticoid response elements (GREs)	↓GR binding to GREs in H-Ras and Raf-1 promoters (chromatin immuno-precipitation) *no ↓binding in vitro	Developing Brain (Mouse)	C57BL/6 mice	50 ppb sodium arsenite (2 wks prior to gestation + through weaning)	(Martinez-Finley et al., 2011)
↓ transcription initiation at GR-regulated promoter	↓reporter gene mRNA initiation	Tumor (Mouse)	1470.2 cells (mouse adenocarcinoma derived)	8 µM sodium arsenite + 5 nM Dex (120 min)	(Barr et al., 2009)
	↓endogenous GR-regulated mRNA (serum glucocorticoid kinase [SGK]) initiation	Tumor (Mouse)	1470.2 cells (mouse adenocarcinoma derived)	8 µM sodium arsenite + 5 nM Dex (120 min)	(Barr et al., 2009)
↑/↓GR mediated gene transcription	↓reporter gene activity (MMTV-chloramphenicol acetyl transferase [MMTV-CAT])	Tumor (Mouse)	1470.2 cells (mouse adenocarcinoma derived)	0.5–8 µM sodium arsenite + 100 nM Dex (4 hr)	(Barr et al., 2009)
	↑ reporter gene activity (G2T-luciferase construct)	Liver (Rat)	EDR3 cells (hepatoma cell line)	<1 µM sodium arsenite + 50 nM Dex (18 hr)	(Bodwell et al., 2006)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
	↓ reporter gene activity (G2T-luciferase construct)			≤ 1–3 µM sodium arsenite + 50 nM Dex (18 hr)	
Mineralocorticoid Receptor (MR)					
↑/↓MR-mediated gene transcription	↑ reporter gene activity (G2T-luciferase construct)	Liver (Rat)	EDR3 cells (hepatoma cell line)	<1 µM sodium arsenite + 0.5 nM aldosterone (18 hr)	(Bodwell et al., 2006)
	↓ reporter gene activity (G2T-luciferase construct)			≤ 1–3 µM sodium arsenite + 0.5 nM aldosterone (18 hr)	
Progesterone Receptor (PR)					
↑/↓PR-mediated gene transcription	↑ reporter gene activity (G2T-luciferase construct)	Liver (Rat)	EDR3 cells (hepatoma cell line)	<1 µM sodium arsenite + 50 nM progesterone (18 hr)	(Bodwell et al., 2006)
	↓ reporter gene activity (G2T-luciferase construct)			≤ 1–3 µM sodium arsenite + 50 nM progesterone (18 hr)	
Thyroid Hormone Receptor (TR)					
Altered TR gene induction	↓TR response element-luciferase (TRE-luc)	Pituitary (Rat)	GH3 rat pituitary tumor cells	0.5–2 µM As(III) + 2 nM Thyroid Hormone (T3) (24 hr)	(Davey et al., 2008)
	↑DIO1	Pituitary (Rat)	GH3 rat pituitary tumor cells	0.1–1 µM As(III) + 2 nM T3 (6 hr)	(Davey et al., 2008)
	↓DIO1			2 µM As(III) + 2 nM T3 (6 hr)	
	↑DIO1			1–2 µM As(III) +2 nM T3 (24 hr)	
Retinoic Acid Receptor (RAR)					
Altered RAR-mediated gene activation	↑Retinoic acid inducible RAR response element (RARE)-luciferase expression induced by all trans-retinoic acid (ATRA)	Embryo (Human)	NTERA-2 (N2) human embryonic carcinoma cells	0.05–0.025 µM As(III) (24 hr)	(Davey et al., 2008)
	↓RARE-luciferase expression induced by ATRA	Embryo (Human)	N2 cells	2.0 µM As(III) (24 hr)	(Davey et al., 2008)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
	↑CYP26A induced by ATRA	Embryo (Human)	N2 cells	0.01 µM As(III) (24 hr)	(Davey et al., 2008)
	↓ CYP26A induced by ATRA			≤ 0.025 µM As(III) (24 hr)	
Alterations in Cell Signaling Pathways Mediated by Hormone Receptors					
Mitogen-activated protein kinase (MAPK) pathway alterations	↓H-Ras & Raf-1 mRNA *no ↓ in protein	Developing Brain (Mouse)	C57BL/6 mice (Postnatal day 35)	50 ppb sodium arsenite (2 weeks prior to gestation + through weaning on postnatal day [PND] 23)	(Martinez-Finley et al., 2011)
	↓phosphorylated-ERK	Developing Brain (hypothalamus; Mouse)	C57BL/6 mice (Postnatal day 35)	50 ppb sodium arsenite (2 wks prior to gestation + through weaning on PND 23)	(Martinez-Finley et al., 2011)
Cellular Responses					
Cytotoxicity	↓colony forming ability	Breast (Human)	Human breast cancer MCF-7 cells	15 µM As(III) (LC50) (24 hr); or 25 µM As(III) (LC50) + 50 pM E2 (24 hr)	(Davey et al., 2007)
			Human breast cancer MCF-7 cells	2 µM ATO + 10 nM 17β-estradiol (IC50) (72 hr) *reduced viability as compared to E2 alone	(Chow et al., 2004)
Cytotoxicity (continued)	↓colony forming ability (continued)	Breast (Human) (continued)	Human breast cancer MCF-7 cells	8 µM ATO (IC50) (24 hr) 1–2 µM ATO (IC50) (72 hr)	(Chow et al., 2004)
			Human breast cancer MDA-MB-231 cells	17 µM ATO (IC50) (24 hr) 4–8 µM ATO (IC50) (72 hr)	(Chow et al., 2004)
		Embryo	NTERA-2 (N2) human embryonic carcinoma cells	3 µM As(III) (LC50) (24 hr)	(Davey et al., 2008)
		Pituitary (Rat)	GH3 rat pituitary tumor cells	5–10 µM As(III) (LC50) (24 hr)	(Davey et al., 2008)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Proliferation	↑colony forming ability	Pituitary (Rat)	GH3 rat pituitary tumor cells	0.01–1 µM As(III) + 10 nM thyroid hormone (T3) (24 hr)	(Davey et al., 2008)
	↑cell number *growth inhibited by antiestrogen	Breast (Human)	Human breast cancer MCF-7 cells	1 µM sodium arsenite (5 – 8 d)	(Stoica et al., 2000)
	↓cell number	Prostate (Human)	LNCaP, or LAPCaP-R1 cells (human prostate cancer cells)	5 µM ATO (3 d and 5 d)	(Rosenblatt and Burnstein, 2009)
Altered cell cycle	21%↓ G1 phase cells 8%↓ S phase cells 12%↓ G2/M phase cells	Breast (Human)	Human breast cancer MCF-7 cells	2 µM ATO (48 hr; greater effect at 72 hr)	(Chow et al., 2004)
	26%↑ G1 phase cells 8%↓ S phase cells 10%↓ G2/M phase cells	Breast (Human)	Human breast cancer MCF-7 cells	2 µM ATO + 10 nM 17β-estradiol (48 hr) *reduced viability as compared to	(Chow et al., 2004)
	↓G1 cell cycle proteins (cyclin D1 and CDK4) mRNA	Uterus (Rat)	Sprague-Dawley rats (Female)	4 µg/mL sodium arsenite (28 d)	(Chatterjee and Chatterji, 2010)
Altered hormone receptor distribution	No change in cytosolic MR protein ↓nuclear MR protein	Developing Brain (Hippo-campus) (Mouse)	C57BL/6 mice (PND 35-40)	55 ppb sodium arsenate (2 wks prior to gestation through PND 23)	(Martinez-Finley et al., 2009)
	↓ cytosolic GR protein ↓nuclear GR protein	Developing Brain (Hippo-campus) (Mouse)	C57BL/6 mice (PND 35-40)	55 ppb sodium arsenate (2 wks prior to gestation through PND 23)	(Martinez-Finley et al., 2009)
	↓ cytosolic GR protein ↑nuclear GR protein	Developing Brain (hypothalamus; Mouse)	C57BL/6 mice (PND 31-40)	50 ppb sodium arsenate (2 wks prior to gestation through weaning on PND 21)	(Goggin et al., 2012)
Tissue or Organ System Responses					
Altered hypothalamic-pituitary-adrenal (HPA) axis activity	↑corticotrophin releasing factor	Developing Brain (hypo-thalamus; Mouse)	C57BL/6 mice (PND 31-40)	50 ppb sodium arsenate (2 wks prior to gestation through weaning on PND 21)	(Goggin et al., 2012)
	↑base-line corticosterone (CORT)	Plasma (Mouse)	C57BL/6 mice (PND 35)	50 ppb sodium arsenate	(Goggin et al., 2012)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
			C57BL/6 mice (PND 75-90)	(2 wks prior to gestation through weaning on PND 21 or 23)	(Martinez et al., 2008)
	↑plasma corticosterone	Plasma (Rat)	Albino rats (Male)	5 mg/kg/d sodium arsenite (6 d/wk for 4 wks)	(Jana et al., 2006)
Altered hypothalamic-pituitary-gonadal (HPG) axis activity	Dose dependent ↓ in: plasma hormone levels (luteinizing hormone [LH], follicle-stimulating hormone [FSH], testosterone;	Plasma (Rat)	Wistar rats (Male)	5 or 6 mg/kg/d sodium arsenite (26 d)	(Sarkar et al., 2003)
	↓in plasma LH, FSH, testosterone	Plasma (Rat)	Albino rats (Male)	5 mg/kg/d sodium arsenite (6 d/wk for 4 wks)	(Jana et al., 2006)
Altered hypothalamic-pituitary-gonadal (HPG) axis activity (continued)	↓serum estradiol levels	Serum (Rat)	Sprague-Dawley rats (Female)	0.4, 4, 40 or 80 µg/mLsodium arsenite (14–56 d)	(Chatterjee and Chatterji, 2010)
	↓ serum LH, FSH levels	Serum (Rat)	Sprague-Dawley rats (Female)	4 µg/mL sodium arsenite (28 d)	(Chatterjee and Chatterji, 2010)
	↓ plasma estradiol, LH, FSH levels *No effects detected at 16 days of exposure	Plasma (Rat)	Sprague-Dawley rats (Female)	0.4 ppm sodium arsenite (16 or 28 d)	(Chattopadhyay et al., 1999)
Testicular toxicity	↓in: paired testicular weights; and testicular testosterone; Altered testicular enzyme levels; germ cell degeneration at stage VII *Effects alleviated by co-administration of human chorionic gonadotrophin **Effects enhanced by co-administration of oestradiol	Male reproductive organs (Rat)	Albino rats (Male)	5 mg/kg/d sodium arsenite (6 d/wks for 4 wks)	(Jana et al., 2006)
	↓ testicular weights, sperm count and motility, altered testicular enzyme activities	Male reproductive organs (Mouse)	Swiss albino mice (Male)	53.39 µmol/L sodium arsenite (365 d)	(Pant et al., 2004)
Impaired Spermatogenesis	Dose dependent ↓ in: reproductive organ weight; epididymal sperm count; and degeneration of germ cells at stage VII	Male reproductive organs (Rat)	Wistar rats (Male)	5 or 6 mg/kg/d sodium arsenite (26 days)	(Sarkar et al., 2003)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Female reproductive toxicity	↓uterine weight; altered uterine morphology	Female reproductive organs (Rat)	Sprague-Dawley rats (Female)	4 µg/mL sodium arsenite (28 d)	(Chatterjee and Chatterji, 2010)
	↓uterine, ovary and vagina weights, ovarian enzymes *No effects detected at 16 days of exposure	Female reproductive organs (Rat)	Sprague-Dawley rats (Female)	0.4 ppm sodium arsenite (16 or 28 d)	(Chattopadhyay et al., 1999)
Altered protein glycosylation	↓fully glycosylated 11β-Hydroxysteroid Dehydrogenase Type 1	Developing Brain (hippo-campus; Mouse)	C57BL/6 mice (PND 75- 90)	50 ppb sodium arsenate (2 wks prior to gestation through weaning on PND 21)	(Goggin et al., 2012)
Altered receptor levels	↑(trend) GR mRNA	Adolescent Brain (hippo-campus; Mouse)	C57BL/6 mice (PND 31-40)	50 ppb sodium arsenate (2 wks prior to gestation through weaning on PND 21)	(Goggin et al., 2012)
	↓corticotrophin-releasing factor receptor	Adult Brain (hippocampus; Mouse)	C57BL/6 mice (PND 75 - 90)	50 ppb sodium arsenate (2 wks prior to gestation through PND 23)	(Martinez et al., 2008)
	↓estrogen receptor mRNA and protein	Uterus (Rat)	Sprague-Dawley rats (Female)	4 µg/mL sodium arsenite (28 d)	(Chatterjee and Chatterji, 2010)
Altered receptor sensitivity	↑specific binding to serotonin receptor (5HT-1A)	Adult Brain (hippo-campus; Mouse)	C57BL/6 mice (PND 75 - 90)	50 ppb sodium arsenate (2 wks prior to gestation through PND 23)	(Martinez et al., 2008)
Altered neurotransmitter levels	↑ dopamine ↓ noradrenaline ↓5-HT	Adult Brain (hypothalamus, pituitary; rat)	Albino rats (Male)	5 mg/kg/d sodium arsenite (6 d/wk for 4 wks)	(Jana et al., 2006)
Impaired morphogenesis	↓T3-dependent tail fin resorption	Tail (Xenopus laevis)	Ex-vivo (Xenopus laevis tails)	0.05–4 µM As(III) + 10 nM T3 (4 d)	(Davey et al., 2008)
Individual Response					
Impaired spatial learning and memory	Novel Object Test ↑time to recognize presence of novel object ↓entries in presence of novel object	Mouse	C57BL/6 mice (PND 35-40)	55 ppb sodium arsenate (2 wks prior to gestation through PND 23)	(Martinez-Finley et al., 2009)
	8-way Radial Arm Maze ↑entry errors				

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Altered stress response	↑base-line corticosterone (CORT) Blunted CORT increase following stressor	Plasma (Mouse)	C57BL/6 mice (PND 35)	50 ppb sodium arsenate (2 wks prior to gestation through weaning on PND 21)	(Goggin et al., 2012)
Depressive like behavior	Learned Helplessness Task ↑latency to escape in	Mouse	C57BL/6 mice (PND 75 - 90)	50 ppb sodium arsenate (2 wks prior to gestation through PND 23)	(Martinez et al., 2008)
	Forced Swim Test ↑immobility	Mouse	C57BL/6 mice (PND 75 - 90)	50 ppb sodium arsenate (2 wks prior to gestation through PND 23)	(Martinez et al., 2008)
Susceptible Individuals					
Developing children	Indicators of developmental neurotoxicity in rodents coupled with lower cogitative performance in epidemiology studies	See rows above and below for animal and epidemiological data, respectively	Rats or human population	Varies	(Martinez-Finley et al., 2009); (Goggin et al., 2012); (Martinez et al., 2008); (Wasserman et al., 2007)
Population Level Response					
Developmental neurotoxicity	↓performance on Wechsler Preschool & Primary Scale of Intelligence	Brain (Human)	6-yr-old children (Araihazar, Bangladesh)	Mean 120.1 µg/L in urine (not specified)	(Wasserman et al., 2007)
Male infertility	Abnormal sperm, ↓ sperm count, sperm mobility	(Human & animal model)	Human and animal models	Varies	(Rosenblatt and Burnstein, 2009)
	↑ male infertility	Reproductive system (Human)	Human population	Varies	(Shen et al., 2013)
Prostate Cancer	↑prostate cancer mortality associated with inorganic arsenic exposures	Prostate (Human)	Human population	Varies	Reviewed in (Prins, 2008)

[a]Exposure duration abbreviations: minutes (min), hours (hr), days (d), weeks (wks), years (yrs).

Table A-4. Preliminary data on effects mediated by epigenetic mechanisms

1 *Relevant Health Effects: Bladder cancer, skin cancer, skin lesions*

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
Molecular Initiating Events					
↓S-adenosyl-methionine (SAM)	SAM depletion associated with methylation, reduction of inorganic arsenic species	Multiple	Multiple	Multiple	Reviewed in (Reichard and Puga, 2010), (Martínez et al., 2011), (Ren et al., 2011b)
↓SAM unrelated to inorganic arsenic methylation	↓SAM in cells with low capacity to methylate inorganic arsenic; ↑expression of transsulfuration enzymes in glutathione (GSH) synthesis	Prostate (Human)	Transformed prostate epithelial cell line (RPWE-1)	5 µM arsenite (16 wks)	(Coppin et al., 2008), Reviewed in (Reichard and Puga, 2010)
↑oxidative stress and subsequent GSH depletion	↑reactive oxygen species (ROS); ↑oxidation of GSH	Multiple	Multiple	Multiple	Reviewed in (Reichard and Puga, 2010)
	transformation of HELF cells via ↑ROS ->ERK/NFKB activation ->hsa-miR-21 upregulation	Embryonic lung (Human)	Embryonic lung fibroblasts (HELF)	1 µM sodium arsenite (up to 30 cell passages)	(Ling et al., 2012)
Biochemical Responses					
Altered DNA methyltransferases (DNMTs) activity	↓ DNMT activity (no change in DNMT mRNA expression), associated with hypomethylation	Prostate (Human)	Human prostate epithelial cells (RWPE-1)	5 µM As(III) (29 wks)	(Benbrahim-Tallaa et al., 2005)
	SAM depletion, ↓ expression of DNMT1 and DNMT3, global hypomethylation	Skin (Human)	Human HaCat keratinocytes	up to 5 µM As(III) (3 d)	(Reichard et al., 2007)
Global DNA methylation changes	Hypermethylation only in folate adequate individuals	Blood (Human)	Peripheral blood lymphocyte (PBL) DNA	2–250 µg/L As(III) (>4 yrs)	(Pilsner et al., 2007)
	Hypermethylation	Blood (Human)	PBL DNA	250–500 µg/L As(III) (>6 mos, mean = 10 yrs)	(Majumdar et al., 2010)
	Hypomethylation	Skin/Blood (Human)	PBL DNA in individuals with skin lesions	2–250 µg/L (As[III]) (>2 yrs)	(Pilsner et al., 2009)
Global DNA methylation changes (continued)	hypomethylation, increased GSH and decreased SAM levels	Prostate (Human)	Human prostate epithelial cells (RWPE-1)	5 µM As(III) (16 wks)	(Coppin et al., 2008)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
	hypomethylation, decreased DNMT activity with no change in DNMT mRNA expression	Prostate (Human)	Human prostate epithelial cells (RWPE-1)	5 µM As(III) (29 wks)	(Benbrahim-Tallaa et al., 2005)
	hypomethylation	Skin (Human)	HaCaT keratinocytes	0.2 µM (4 wks)	(Reichard et al., 2007)
	hypomethylation	Liver (Rat)	Rat liver epithelial cells (TRL 1215)	125–500 nM As(III) (18 wks)	(Zhao et al., 1997)
	hypomethylation (after 1 day) and chromosomal instability (8 weeks)	Lung (Hamster)	Chinese hamster cells (V79-CI3)	10 µM As(III) (1 day – 8 wks)	(Sciandrello et al., 2004)
	hypomethylation, increased expression of ERα and cyclin CD1 mRNA and protein	Liver (Mouse)	129/SvJ mice	45 ppm As(III) (48 wks)	(Chen et al., 2004)
	hypomethylation, gene expression changes	Liver (Mouse)	Homozygous Tg.AC mice	150 ppm As(III); 200 ppm As(V); 1,500 ppm MMA(V); or 1,200 ppm DMA(V) (17 wks)	(Xie et al., 2004)
	hypomethylation; correlation with c-myc gene expression, tumor formation in nude mice	Liver (Rat)	Rat liver epithelial cells (TRL 1215)	125–500 nM As(III) (18 wks)	(Chen et al., 2001)
	hypo and hypermethylation	Kidney and lung (Human)	kidney (UOK) and lung epithelial type II (A549) cell lines	As(III) (various)	(Zhong and Mass, 2001)
	Altered methylation patterns in repetitive DNA elements (high in Alu and low in LINE-1 with higher inorganic arsenic exposure)	Blood (Human)	elderly men; blood leukocyte DNA methylation	0.02–1.45 µg/g toenail arsenic (unspecified)[b]	(Lambrou et al., 2012)
Global DNA methylation changes (continued)	↑Global methylation	Brain cortex and hippocampus (Rat)	Wistar Rats	3 ppm sodium arsenite; or 36 ppm sodium arsenite (10 days prior to gestation through 1-month postnatal development)	(Martínez et al., 2011)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
	Hypomethylation	Brain cortex (Rat)	Wistar Rats	3 ppm sodium arsenite; or 36 ppm sodium arsenite (10 days prior to gestation through 3 or 4 months postnatal development)	(Martínez et al., 2011)
Gene specific methylation changes	182 hypermethylated genes (17 = tumor suppressor); 1 hypomethylated gene	Skin and Blood (Human)	PBL DNA (Zimapan, Mexico)	110 µg As/L (mean) (>2 yrs)	(Smeester et al., 2011)
	Aberrant DNA methylation; cellular transformation	Bladder (Human)	Human bladder cell line (UROtsa)	50 nM MMA(III) (12, 24 wks)	(Wnek et al., 2010)
	altered DNA methylation of 455 promoters (primarily hypomethylation), associated with urinary iAs	Urine and blood (Human)	Human Urine (16 females in Zimapan, Hildago, Mexico)	3.6–31.8 ng Total As/mL in urine (10.7 ng/mL [mean]) (unspecified)	(Bailey et al., 2013)
	DAPK promoter hypermethylation	Bladder (Human)	Human bladder, kidney, ureter tumors from urothelial carcinoma patients (Southwest Taiwan)	Unspecified high doses from well water (unspecified)	(Chen et al., 2007a)
	p53, p16 promoter hypermethylation (dose-dependent), hypomethylation in highest exposure group	Blood (Human) associated with skin lesions	Human PBL (West Bengal, India)	>50 µg/L As in drinking water (≤ 6 mos) highest group: 300–1,000 As µg/L in drinking water (≤ 6 months)	(Chanda et al., 2006)
Gene specific methylation changes (continued)	p16 promoter hypermethylation	Blood (Human)	Human PBL in patients with arseniasis (Guizhou Province, China)	Unspecified doses from use of unventilated coal stove with high As (unspecified)	(Zhang et al., 2007)
	RASSF1A, PRSS3 promoter hypermethylation	Bladder (Human)	Human Bladder tumors (New Hampshire, U.S.)	>0.26 µg/g toenail As (unspecified)	(Marsit et al., 2006b)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
	DBC1, FAM83A, ZSCAN12, C1QTNF6 promoter hypermethylation	Bladder (Human)	UROtsa urothelial cells	1 µM As(III), or 50 nM MMA(III) (52 wks)	(Jensen et al., 2008)
	WNT5A promoter hypermethylation	Bladder (Human)	UROtsa urothelial cells	1 µM As(III), or 50 nM MMA(III) (52 wks)	(Jensen et al., 2009b)
	DAPK promoter hypermethylation and reduced expression	Bladder (Human)	Uroepithelial cells (SV-HUC-1)	2,4,10 µM As(III) (2 d)	(Chai et al., 2007)
	p16 promoter hypermethylation	Immune System (Human)	Myeloma cells (U266)	1,2 µM As_2O_3 (3 d)	(Fu and Shen, 2005)
	p53 promoter hypermethylation	Lung (Human)	Lung adenocarcinoma cells (A549)	0.8–2 µM As(III), or 30–300 µM As(V) (1 wk)	(Mass and Wang, 1997)
	c-myc, c-Hras promoter hypomethylation	Embryo (Hamster)	Syrian hamster embryo cells	3–10 µM As(III), or 50–150 µM As(V) (2 d)	(Takahashi et al., 2002)
	p16, RASSF1 promoter hypermethylation, ↓ expression of p16 and RASSF1, increased occurrence of lung adenocarcinoma	Lung (Mouse)	A/J mice	1, 10, 100 ppm As(V) (18 ms)	(Cui et al., 2006a)
	p16, RASSF1A, E-cadherin, GSTP1 promoter hypomethylation	Liver (Human)	HepG2 and Huh-7 liver cells	2–10 µM As(III) (3 d)	(Cui et al., 2006b)
	c-Hras promoter hypomethylation in dietary methyl deficient mice, steatosis and microgranulomas	Liver (Mouse)	C57BL/6J mice	2.6–14.6 µg As(III)/g body weight/day (18.5 wks)	(Okoji et al., 2002)
Gene specific methylation changes (continued)	ERα promoter hypomethylation	Liver (Mouse)	C3H mice (Adult male with hepatocellular carcinoma [HCC] after only in utero exposure)	85 ppm As(III) (gestational day [GD] 8 – 18)	(Waalkes et al., 2004a)
	ERα promoter hypomethylation, ↑ expression of ERα and cyclin CD1 mRNA and protein	Liver (Mouse)	129/SvJ mice	45 ppm As(III) (48 wks)	(Chen et al., 2004)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
	hyper- and hypomethylation of VHL promoter	Kidney (Human)	Human kidney cells (UOK123, UOK109, UOK121)	IC_{30}, IC_{50}, or IC_{80} of each cell line: 7 – 93 µM As(III) (4 wks)	(Zhong and Mass, 2001)
Histone modification	↓ acetylation (H3K18ac) ↓methylation (H3R17me)	Tumor (Mouse)	1470.2 cells (mouse adenocarcinoma derived)	8 µM sodium arsenite + 5 nM Dex (15 min)	(Barr et al., 2009)
	↑ histone acetylation (H3; lysine 14) and phosphorylation (H3; serine 10) at c-jun and c-fos chromatin, increased expression of c-jun and c-fos	Lung (Human)	Human fibroblasts (WI-38 cells)	400 µM As(III), (up to 1 hr)	(Li et al., 2003)
	↑ histone H3acetylation (H3K9); inhibition of HDAC activity	Liver (Human)	Human hepatoma HepG2 cells	5–10 µM As(III) (1 d)	(Ramirez et al., 2008)
	↓ histone acetylation: H4K16, H3K9, K14, K18, K23	Bladder (Human)	Human uroepithelial cells (UROtsa)	1–10 µM As(III) or 0.3–3 µM MMA(III) (up to 1 d)	(Chu et al., 2011)
	↓ H4; lysine 16 acetylation	Bladder (Human)	Human bladder epithelial cells (UROtsa)	150 µM As(III), or 300 µM MMA(III) (1 d)	(Jo et al., 2009)
	↓ H3 acetylation of FAM83A, DCB1, ZSCAN12, KRT7, C1QTNF6, FGF5; increased acetylation of KCNK10, NEFL	Bladder (Human)	UROtsa and URO-ASSC urothelial cells	1 µM As(III), or 50 nM MMA(III) (52 wks)	(Jensen et al., 2008)
Histone modification (continued)	↑permissive transcription histone modifications (DiMeK4; AcH3) ↓repressive transcription histone modifications (TriMeK27, DiMeK9)	Bladder (Human)	UROtsa and URO-ASSC urothelial cells	1 µM As(III), or 50 nM MMA(III) (52 wks)	(Jensen et al., 2009b)
	↓ H3K27 trimethylation, ↑ H3K9 dimethylation and H3K4 trimethylation (increase in HMT G9a protein and mRNA levels)	Lung (Human)	A549 human lung adenocarcinoma cells	0.1–10 µM As(III) (1 d)	(Zhou et al., 2008)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
	↑ H3K4 trimethylation, maintained after inorganic arsenic removal = inherited through cell division	Lung (Human)	A549 human lung adenocarcinoma cells	0.1–1 µM As(III) (1 or 7 d)	(Zhou et al., 2009)
	↑ H2AX phosphorylation	Skin (Human)	Melanoma cells (RPMI7591)	1, 2.5, or 5 µM As(III) (1 day)	(Zykova et al., 2006)
	↑ H3K9me2 and ↓ H3K9ac with increased urinary inorganic arsenic; other histone marks correlated with water inorganic arsenic in gender specific manner	Blood (Human)	Peripheral blood mononuclear cells (Bangladesh cohort [n=40])	91.5 µg/L urinary inorganic arsenic (median) (unspecified)	(Chervona et al., 2012); (Arita et al., 2012)
	↑ H3K9me2; ↓ p16INK4a expression; no change in promoter DNA methylation	Liver (Mouse)	C57Bl/6J mice	50 ppm sodium arsenite (6 mos)	(Suzuki and Nohara, 2013)
Altered MicroRNA expression	Upregulation of hsa-miR-22,34a,221,222 and downregulation of hsa-miR-210	Immune system (Human)	Human immortalized lymphoblast cells (TK6 cell line)	≤ 2 µM As(III) (6 d)	(Marsit et al., 2006a)
	downregulation of miRNA-19a - cell growth arrest and apoptosis	Bladder (Human)	T24 human bladder carcinoma cells	4 µM As_2O_3 (24 hr)	(Cao et al., 2011)
Altered MicroRNA expression (continued)	upregulation of hsa-miR-2909; molecular responses linked to immune response	Immune system (Human)	Peripheral blood mononuclear cells (PBMCs)	2 µM sodium arsenite (48 hr)	(Kaul et al., 2014)
	85 miRNA upregulated, 52 downregulated; predicted to be involved in regulating phosphoproteins and alternative gene splicing	Vascular system (Human)	Umbilical vein endothelial cells (HUVECs)	20 µM sodium arsenite (24 hr)	(Li et al., 2012)
	hsa-miR-21 upregulation	Embryonic, lung (Human)	Embryonic lung fibroblast (HELF)	1 µM sodium arsenite (up 30 cell passages)	(Ling et al., 2012)
Cellular Phenotypic Changes					

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
Malignant transformation	transformation of HELF cells via increased ROS ->ERK/NFKB activation ->hsa-miR-21 upregulation	Embryonic, lung (Human)	Embryonic lung fibroblast (HELF)	1 µM sodium arsenite (up 30 cell passages)	(Ling et al., 2012)
	transformation of p53 knocked down HBECs; downregulated hsa-miR-200b via promoter methylation	Lung (human)	p53(low) human bronchial epithelial cells	2.5 µM sodium arsenite (16 wks)	(Wang et al., 2011b)
	Altered H3 and H4 acetylation during malignant transformation	Bladder (Human)	UROtsa and URO-ASSC urothelial cells	1 µM As(III), or 50 nM MMA(III) (52 wks)	(Jensen et al., 2008)
	Increase in "permissive" histone modifications AcH3 and DiMeK4; repressive modifications TriMeK27 and DiMeK9 were decreased à non-canonical WNT5A signaling and malignant transformation	Bladder (Human)	UROtsa and URO-ASSC urothelial cells	50 nM MMA(III) (24+ wks)	(Jensen et al., 2009b)
Malignant transformation (continued)	Genome-wide changes in promoter DNA methylation, increasing with duration of exposure, in parallel with phenotypic changes (transformation)	Bladder (Human)	UROtsa and URO-ASSC urothelial cells	1 µM As(III), or 50 nM MMA(III) (up to 52 wks)	(Jensen et al., 2009a)
Tissue/Organ Responses					
Skin Lesions	Development of skin lesions associated with inorganic arsenic exposure and PBL hypomethylation	Skin/Blood (Human)	PBL DNA in individuals with skin lesions (Araihazar, Bangladesh)	121 µg/L urinary As (>2 yrs)	(Pilsner et al., 2009)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
	Risk of skin lesions associated with DAPK and p16 hypermethylation	Skin and blood (Human)	PBL DNA in individuals (West Bengal, India)	567.25 µg/L mean urinary As(III) (with lesions) Mean urine As(III) 495.48 µg/L mean urinary As(III) (w/o lesions), 567.25 µg/L (with lesions)	(Banerjee et al., 2013)
Adverse liver effects	Hepatic steatosis with DNA hypomethylation	Liver (Mouse)	129/SvJ mice	45 ppm As(III) (48 wks)	(Chen et al., 2004)
	Hepatocellular carcinoma	Liver (Mouse)	Adult male C3H mice with HCC after only in utero exposure	85 ppm As(III) (gestational day [GD] 8–18)	(Waalkes et al., 2004a)
	Steatosis and microgranulomas with c-Hras promoter hypomethylation in dietary methyl deficient mice	Liver (Mouse)	C57BL/6J mice	2.6–14.6 µg As(III)/g body weight/d (18.5 wks)	(Okoji et al., 2002)
Individual Responses					
Contextual memory deficits	↓freezing behavior *highest dose group: significant at all time points 2 -4 months of age Lowest dose group: significant at 1 time point at 2 months of age; all time points 3 & 4 months of age	Whole animal (Rat)	Wistar Rats	3 or 36 ppm sodium arsenite, (10 d prior to gestation through 1–2, or 3– 4-mos postnatal development)	(Martínez et al., 2011)
Susceptible Individual response					
Diet (e.g., deficiencies in methyl, folate, methionine)	Altered DNA methylation patterns in repetitive Alu and LINE DNA elements (high Alu methylation correlated with high inorganic arsenic exposure in low folate condition, and vice versa) following low levels of environmental exposure	Blood (Human; Elderly men)	Blood leukocyte DNA in human cohort study	0.02–1.45 µg/g toenail arsenic (unspecified)	(Lambrou et al., 2012)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
	Hypermethylation, modified by folate	Blood (Human)	PBL DNA	2–250 µg/L As(III) (>4 yrs)	(Pilsner et al., 2007)
	development of skin lesions associated with low folate	Skin/Blood (Human)	PBL DNA in individuals with skin lesions	2–250 µg/L As(III) (>2 yrs)	(Pilsner et al., 2009)
	c-Hras promoter hypomethylation, steatosis and microgranulomas	Liver (Mouse)	C57BL/6J mice	2.6–14.6 µg iAs (III)/g body weight/d (18.5 wks)	(Okoji et al., 2002)
	5357 CpG islands altered with high maternal folate + inorganic arsenic	Fetal liver (Mouse)	CD-1 mice (Pregnant females)	85 ppm As(III) (GD 8–18) + High maternal folate intake (11 mg/kg) (GD 5–18)	(Tsang et al., 2012)
Life stage (in utero exposure)	global hypomethylation w/ high exposure, PP1 promoter hypomethylation, reduced fear memory	Brain (rat)	Wistar rats	3 or 36 ppm sodium arsenite (gestation to 4 mos postnatal development)	(Martínez et al., 2011)
	ERα promoter hypomethylation, HCC	Liver (Mouse)	C3H mice (Adult; male)	85 ppm As(III) (GD 8 – 18)	(Waalkes et al., 2004a)
	12 miRNAs upregulated (linked to cancer, diabetes and immune response signaling pathways)	Blood (Human)	cord blood (Mexican women's cohort)	0.456–236 µg/L inorganic arsenic in maternal drinking water inorganic arsenic range of 0.456–236 µg/L; maternal urine inorganic arsenic range of 6.2–319.7 µg/L inorganic arsenic in maternal urine (unspecified)	(Rager et al., 2014)
	5357 CpG islands altered with high maternal folate + inorganic arsenic	Fetal liver (Mouse)	CD-1 mice (Pregnant females)	85 ppm As(III) (GD 8–18) + High maternal folate intake (11 mg/kg) for (GD 5–18)	(Tsang et al., 2012)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References
Gender	Males: ↓DNA methylation; ↓DNMT1 expression (no change in SAM content) Females: ↑DNA methylation in females (no change in DNMT1 levels) ↓SAM content	liver (Mouse)	C57BL/6J mice	50 ppm sodium arsenite + methyl-deficient diet ad libitum (5 mos)	(Nohara et al., 2011)
Genetics	AS3MT haplotype associated with efficient inorganic arsenic metabolism, methylation of AS3MT gene region and reduced AS3MT mRNA expression	Blood / Skin (Human)	Human peripheral blood (Argentinian women)	188 µg/L mean total urinary arsenic (unspecified)	(Engström et al., 2013)
Population response					
Hypermethylation of genes related to diseases associated with inorganic arsenic (e.g., cancer, heart disease, diabetes)	182 hypermethylated genes related to tumor suppression (e.g., forkhead box F1 [FoxF1], matrix metallopeptidase 15 [MMP15])	Peripheral blood lymphocytes (Human)	Females (n= 8) with inorganic arsenical skin lesions in Zimapan, Hidalgo State, Mexico; compared to females (n=8) without lesions	63.47 µg/g total arsenic in urinary creatinine (average) (unspecified)	(Smeester et al., 2011)
Inorganic arsenic induced bladder cancer risk	Promoter methylation silencing of tumor suppressor genes (p16, RASSF1A, PRSS3) and soluble Frizzled receptor proteins (SFRPs) in 30-50% of bladder cancer cases	Bladder tumors (Human)	Participants in population-based case-control of bladder cancer in New Hampshire, U.S.	≤ 0.26 µg/g toenail arsenic (unspecified)	(Marsit et al., 2006c); (Marsit et al., 2006b)
Inorganic arsenic-induced skin cancer risk	Dose-related increase in hypermethylation of p53 gene in inorganic arsenic exposed individuals compared to controls & individuals with inorganic arsenic-induced skin cancer patients	Blood (Human)	Human subjects in Kolkata, India (individuals with inorganic arsenic associated skin cancer & non arsenic cancer)	Controls: <50 µg/L inorganic arsenic in drinking water Exposed: 51–1,000 µg/L inorganic arsenic in drinking water (9.5 – 19 yrs)	(Chanda et al., 2006)
Inorganic arsenic induced skin lesions	development of skin lesions associated with low folate	Skin/Blood (Human)	PBL DNA in individuals with skin lesions	2–250 µg/L As(III) (>2 yrs)	(Pilsner et al., 2009)

Key events	Observations	Observation organ system	Test system observed in	Dose (exposure duration)[a]	References

[a]Abbreviations used for exposure durations: minutes (min), hours (hr), days (d), weeks (wks).
[b]Exposure durations are characterized as "unspecified" when a study does not explicitly state the exposure duration.

Table A-5. Preliminary data on effects mediated by the immune system

Relevant Health Effects: Suppression of humoral immunity (i.e., decreased antibody response), Suppression of innate immunity (decreased macrophage function), Respiratory infection, Gastrointestinal infection, Contact hypersensitivity response

Key Events	Observations	Organ system	Test System	Dose (Exposure Duration)[a]	References
Molecular Initiating Events					
Molecular initiating events for inorganic arsenic immunotoxicity are unknown. There is some suggestion that generation of ROS may lead to some immune effects. For example, T-cell apoptosis appears through a ROS-dependent pathway [e.g., (Park et al., 2003a); (Gupta et al., 2003)] There is also evidence the effects on macrophages (and therefore many of the effects on the innate immune system which are associated with macrophage function) are unrelated to increased production of ROS. For example, arsenic trioxide alters macrophage gene expression through a pathway independent of ROS production, and EGR2 may be one of the molecular targets of inorganic arsenic (Bourdonnay et al., 2009)					
Biochemical Responses					
↓ATP-mediated Ca(2+) signaling	↓wound-induced healing and peak Ca(2+)	Lung (Human)	Immortalized human bronchial epithelial cells (16HBE14o-) in vitro	0, 130, or 330 nM arsenic as sodium arsenite (4–5 wk)	(Sherwood et al., 2013)
	↓wound-induced total Ca(2+) signaling	Lung (Mice)	C57Bl6 male mice ex vivo	50 ppb sodium arsenite drinking water (4 wk)	(Sherwood et al., 2013)
	↓wound-induced healing, Ca(2+), and # cells in Ca(2+) wave	Lung (Human)	Immortalized human bronchial epithelial cells (16HBE14o-) in vitro	0.8 or 3.9 µM sodium arsenite (24 hr)	(Sherwood et al., 2011)
↓ production of interleukin-2 (IL-2), interferon-gamma (IFN-gamma)	↓IL-2, ↓IFNγ, and ↓IL-4 secreted protein from splenocytes in culture, ConA or anti-CD3 stimulated	Spleen (Mice)	C57Bl6 male mice in vitro [young or aged mice (IL-10 also ↓ from old mice)]	0, 0.03, 0.06, 0.13, 0.25, 0.50, 1, 2 µM Sodium arsenite (48 hr)	(Cho et al., 2012)
	↓IL-2, ↓IFNγ, ↓ IL-4 and ↓IL-12 secreted protein from splenocytes in culture, ConA or anti-CD3/CD28 stimulated	Spleen (Mice)	Male C57Bl/6N mice in vivo	0, 0.01, 0.1, 1 mg/kg sodium arsenite i.g. (30 d)	(Soto-Peña and Vega, 2008)
	↓IL-2, ↓IFNγ secreted protein and mRNA level from splenocytes in culture anti-CD3/CD28 stimulated	Primary T-cells (Human)	Human T-cells from PBMCs from healthy donors in vitro	0, 0.25, 0.50, 1, 2 µM Sodium arsenite (6 or 24 hr)	(Morzadec et al., 2012)

Key Events	Observations	Organ system	Test System	Dose (Exposure Duration)[a]	References
↓ production of interleukin-2 (IL-2), interferon-gamma (IFN-gamma) *(continued)*	↓IL-2 secreted protein levels from PHA-stimulated mononuclear cells; no difference in IFNγ, IL-4, IL-10	Primary monocytes differentiated in 6 days into macrophages (Human)	Human monocytes from PBMCs from children (6-10) living in central Mexico chronically exposed to As in drinking water	Low exposure group: Girls: 11.8 µg/l Boys: 15.6 µg/l urinary As (mean) High exposure group: Girls: 88.2 µg/L Boys: 84.4 µg/L) urinary As (mean) (unspecified)[b]	(Soto-Peña et al., 2006)
	↓IL-2 secreted protein levels from PHA-stimulated mononuclear cells	Primary mononuclear cells (Human)	Human PBMCs from healthy donors in vitro	0, 0.01, 0.1, 1 µM Sodium arsenite (24–48 hr)	(Galicia et al., 2003)
	↓IL-2 secreted protein level PHA-stimulated mononuclear cells	Primary mononuclear cells (Human)	Human PBMCs from healthy donors in vitro	0, 0.01, 0.1, 1 µM Sodium arsenite (24–48 hr)	(Vega et al., 1999)
	↓IL-2 at protein and mRNA level ↓IL-2 splenocytes in culture, PHA-stimulated	Spleen (Mice)	C57Bl6 female mice in vitro	0, 1, 10 µM sodium arsenite (12, 24, 48 hr)	(Conde et al., 2007)
	↓IL-2, ↓IFNγ, ↓IL-4, ↓TNFα, ↓IL-10, ↓IL-5 secreted protein in culture, ConA stimulated	Primary T-cells (Human)	Human T-cells from PBMCs from exposed and unexposed donors	20 individuals with skin lesions compared to 18 unexposed	(Biswas et al., 2008)
	↓IL-2 secreted protein from splenocytes in culture, and both↓IL-2 ↓IFNγ at mRNA level, ConA stimulated	Spleen (Chicken)	Chicken in vitro	1 and 10 µM sodium arsenite (24, 48, 72 hr)	(Das et al., 2011)
↓proliferation of lymphocytes	↓ConA-stimulated T-cell proliferation in culture [^{3}H] TdR incorporation	Primary T-cells (Human)	Human T-cells from PBMCs from exposed and unexposed donors	20 individuals with skin lesions compared to 18 unexposed	(Biswas et al., 2008)
	↓ConA-stimulated T-cell proliferation in culture [^{3}H] TdR incorporation	Spleen (Rats)	Male Wistar rats in vivo	25 ppm sodium arsenite in drinking water (42 d)	(Sankar et al., 2013)
	Slower proliferation response to PHA- T-cell in culture [^{3}H] TdR incorporation	Primary T-cells (Human)	Human T-cells from PBMCs from exposed (33 individuals from an area in Mexico) and unexposed (30) donors	Exposed: 412 µg/L As in water (758 ± 364 µg/L total As in urine) Unexposed: 37 µg/L in water (37 ± 37 µg/L total As in urine)	(Gonsebatt et al., 1994)

Key Events	Observations	Organ system	Test System	Dose (Exposure Duration)[a]	References
↓proliferation of lymphocytes *(continued)*	↓PHA-stimulated T-cell proliferation in culture [^{3}H] TdR incorporation	Primary mononuclear cells (Human)	Human PBMCs from healthy donors in vitro	0, 0.01, 0.1, 1µM Sodium arsenite (24–48 hr)	(Vega et al., 1999)
	↓PHA-stimulated T-cell proliferation in culture [^{3}H] TdR incorporation	Primary monocytes differentiated in 6 days into macrophages (Human)	Human monocytes from PBMCs from children (6-10) living in central Mexico chronically exposed to As in drinking water	Low exposure group: Girls: 11.8 µg/L Boys: 15.6 µg/L urinary As (mean) High exposure group: Girls: 88.2 µg/L Boys: 84.4 µg/L) urinary As (mean)	(Soto-Peña et al., 2006)
Cell signaling change	NF-κB (↑phosphorylated p65)	Lung (Mice)	Nrf2-WT and Nrf2-KO mice in vivo	0.48 mg/m^3 synthetic dust [10% arsenic trioxide + inert dust] (30 min/d/14 d)	(Zheng et al., 2012)
	NF-κB (↓ DNA binding of p65 NF-κB)	Primary monocytes and pro-monocyte cell line (Human)	Human monocytes from PBMCs from healthy donors and pro-monocytic U937 cell line in vitro	0.25–1 µM arsenic trioxide (1, 2, 3, 4, 6 d)	(Lemarie et al., 2006)
	↓enzymatic activity of lysosomal protease cathepsin L	Primary lymphocytes (Human)	Human PBMCs from blood of healthy volunteers In vitro	0, 1, 2, 3, 4, 5µM arsenic trioxide (48 hr)	(Gupta et al., 2003)
	↓transcription factor ERG2	Primary monocytes differentiated into macrophages (Human)	Human monocytes from PBMCs from healthy donors in vitro	1µM arsenic trioxide (48, 72 hr)	(Bourdonnay et al., 2009)
	↑basal phosphorylation of Lck and Fyn kinases and ↑ phosphorylation of Lck and Fyn after stimulation by antibodies to CD3/CD28 in splenocytes	Spleen (Mice)	Male C57Bl/6N mice in vivo	0, 0.01, 0.1, 1 mg/kg sodium arsenite intra-gastric (30 d)	(Soto-Peña and Vega, 2008)
Cellular Phenotypic Changes					
↓monocyte/ macrophage activity or number	↓monocyte recruitment to peritoneal cavity following thioglycollate stimulation	Macrophages (Mice)	Female balb/c mice in vivo	50 mg/L sodium metaarsenite in drinking water (4 wks)	(Patterson et al., 2004)

Key Events	Observations	Organ system	Test System	Dose (Exposure Duration)[a]	References
	↓differentiation monocytes to macrophages by expression of transferrin receptor CD71	Primary monocytes and pro-monocyte cell line (Human)	Human monocytes from PBMCs from healthy donors and pro-monocytic U937 cell line in vitro	0.25–1 µM arsenic trioxide (1, 2, 3, 4, 6 d)	(Lemarie et al., 2006)
	↑apoptosis of splenic macrophages indicated by DNA fragmentation	Spleen (Mice)	Male Swiss albino mice in vivo	0.5 sodium arsenite mg/kg bw/d (15 d)	(Sengupta and Bishayi, 2002)
	↑apoptosis of monocytes, macrophages by Annexin V-Alexa568 (A5) and SG co-staining	Primary monocytes and pro-monocyte cell line (Human)	Human monocytes from PBMCs from healthy donors and pro-monocytic U937 cell line in vitro	0.25–1 µM arsenic trioxide (1, 2, 3, 4, 6 d)	(Lemarie et al., 2006)
	↑ basal apoptosis of monocytes, macrophages by PI staining and analysis for hypodiploid cells	Primary monocytes (Human)	Human monocytes from children chronically exposed to As and nearby unexposed children	Urinary As range from 94 to 240 µg/g-creatinine exposed children living in town near gold mine, 17–34 µg/g-creatinine in nearby unexposed children	(de la Fuente et al., 2002)
	↑ apoptosis of monocytes, macrophages by DNA content assay, Annexin V binding, DNA fragmentation, TUNEL	Primary monocytes (Human)	Human monocytes from PBMCs from healthy donors in vitro	0, 1, 5, 15,30, 50, 75, 100 µM sodium arsenite (12, 36, 48, 72 hr)	(de la Fuente et al., 2002)
↓monocyte/ macrophage activity or number *(continued)*	↑macrophage cell rounding, ↓adhesion, ↓phagocytosis of *S. typhimurium* in 3h, ↓NO^- and O_2^- following LPS stimulation overnight	Primary monocytes differentiated in 6 days into macrophages (Human)	Human monocytes from PBMCs from individuals) in West Bengal India with skin lesions (n= 70) chronically exposed to As in drinking water (Murshidabad) and unexposed (n=64) (West Midnapore	Exposed individuals: 50 to 1,200 µg/L arsenic in drinking water levels Unexposed individuals: levels 3 to 10 µg/L inorganic arsenic in drinking water	(Banerjee et al., 2009)
	Splenic macrophages ↓NO^- and O_2^- after LPS stimulation, ↓macrophage adhesion, ↓chemotaxis, ↓phagocytosis of SRBCs	Spleen (Mice)	Male Swiss albino mice in vivo	0.5 sodium arsenite mg/kg bw/d (15 d)	(Sengupta and Bishayi, 2002)

Key Events	Observations	Organ system	Test System	Dose (Exposure Duration)[a]	References
	↓macrophage phagocytosis of *A. hydrophila*	Macrophages (Catfish)	Catfish in vivo	42.42 µM arsenic trioxide (21 d)	(Ghosh et al., 2006)
	↑macrophage abnormal morphology, ↓adhesion, ↓chemotaxis	Spleen (Mice)	Male Swiss albino mice in vivo	0.5 mg/kg bw (intraperitoneal injection) sodium arsenite (15 d)	(Bishayi and Sengupta, 2003)
	↓ monocyte /macrophage ROS after PMA, ↓ NO^- after RD-F or LPS stimulation	Primary monocytes (Chicken)	Chicken monocytes from PBMCs in vivo	3.7 ppm sodium arsenite in drinking water (10, 20, 30, 40, 60 d)	(Aggarwal et al., 2008)
	↓ monocyte /macrophage ROS after PMA, ↓ NO^- after LPS stimulation	Spleen and peritoneal macrophages (Mice)	Female c57BL7J/Han mice	0, 0.5, 5, 50 sodium hydrogen arsenate (12 wks)	(Arkusz et al., 2005)
	↑apoptosis of monocytes, macrophages by Annexin V—FITC and PI staining	Primary monocytes and pro-monocyte cell line (Human)	Human monocytes pro-monocytic U937 cell line in vitro	0, 0.5, 1, 2.5, 5µM tetraarsenic oxide and diarsenic oxide (0, 2, 4, 6, 8, 10, 12 hr)	(Park et al., 2003b)
↑neutrophil apoptosis	↑neutrophil apoptosis determined by CD16 shedding independent of MAPKs	Human (Lung)	Human neutrophils from venous blood of healthy volunteers In vitro	5 µM arsenic trioxide (.25–180 min)	(Binet and Girard, 2008)
↑T-lymphocyte apoptosis	↑T-cell apoptosis determined by TUNEL assay	Primary lymphocytes (Human)	Human PBMCs from blood of healthy volunteers In vitro	0, 1, 2, 3, 4, 5 µM arsenic trioxide (48 hr)	(Gupta et al., 2003)
↑ B-lymphocyte apoptosis	↑B-cell apoptosis determined by Annexin V assay	Lymphocytes (Mice)	Mouse B cell lymphoma line TA3 In vitro	0, 0.8, 4, 20, 100, 500 µM sodium arsenite (18 hr)	(Harrison and Mccoy, 2001)
↓Langerhans cell migration	↓activated Langerhans cells in cervical lymph nodes of DNFB-sensitized mice by fluorescence-activated sorting	Immune, Skin (Mice)	Female balb/c mice in vivo	50 mg/l sodium metaarsenite in drinking water (4 wks)	(Patterson et al., 2004)
Tissue/ Organ Responses					
↓Thymus size	↓Thymus size assessed sonographically	Thymus (Human)	Children in Matlab region of Bangladesh cohort	Maternal arsenic metabolites in urine at weeks 8 and 30 of gestation	(Raqib et al., 2009); (Moore et al., 2009)

Key Events	Observations	Organ system	Test System	Dose (Exposure Duration)[a]	References
	↓absolute, not relative thymus weight	Thymus (Chicken)	Chickens in vivo	3.7 ppm sodium arsenite in drinking water (10, 20, 30, 40, 60 d)	(Aggarwal et al., 2008)
Individual Responses					
↓delayed-type hypersensitivity (DTH) response	↓DTH to KLH by footpad thickness	Immune function (Rats)	Male Wistar rats in vivo	25 ppm sodium arsenite in drinking water (42 d)	(Sankar et al., 2013)
	↓DTH to 2,4-dinitro-1-chlorobenzene (DNCB) or PHA-P by skin thickness	Immune function (Chicken)	Chickens in vivo	3.7 ppm sodium arsenite in drinking water (10, 20, 30, 40, 60 d)	(Aggarwal et al., 2008)
	↓phytohemagglutinin hypersensitivity response by skin thickness	Immune function (Rats)	Male cotton rats	0, 5, 10 ppm sodium arsenite (6 wks)	(Savabieasfahani et al., 1998)
↓antibody response	↓decreased antibody response by ELISA to vaccination with disease virus (F-strain; RD-F)	Immune function (Chicken)	Chickens in vivo	3.7 ppm sodium arsenite in drinking water (10, 20, 30, 40, 60 d)	(Aggarwal et al., 2008)
↓antibody response *(continued)*	↓antibody response by agglutination to bacterial (*A. hydrophila*) challenge; ↓antigen-specific plaque-forming cells to SRBC	Immune function (Catfish)	Catfish in vivo	42.42 µM arsenic trioxide (150 d)	(Ghosh et al., 2007)
	↓antibody response to SRBC by PFC	Immune function (Mice)	Male c57bl/6N mice in vivo	50 µg/m^3 and 1 mg/m^3 nose only inhalation arsenic trioxide (14 d)	(Burchiel et al., 2009)
	↓antibody response for IgG at day 14 to KLH by ELISA; not significant in IgM at day 5	Immune function (Mice)	Male Wistar rats in vivo	0, 0.4, 4, 40 ppm sodium arsenite in drinking water (18 wks)	(Nain and Smits, 2012)
	↓antibody response to SRBC by PFC	Immune function (Mice)	Male white Swiss cross mice in vivo	0, 0.5, 2, 10 ppm sodium arsenite in drinking water (3 wks)	(Blakley et al., 1980)
↓host resistance (to infection)	↓ ability to decrease bacteria load (*C. batrachus*), ↑tissue damage, slower recovery, ↑mortality	Immune function (Catfish)	Catfish in vivo	42.42 µM arsenic trioxide (150 d)	(Ghosh et al., 2007)
	↓ blood and splenic clearance bacterial (*S. aureus)* challenge	Immune function (Mice)	Male Swiss albino mice in vivo	Sodium arsenite (p.5 mg/kg bw (ip) (15 d)	(Bishayi and Sengupta, 2003)

Key Events	Observations	Organ system	Test System	Dose (Exposure Duration)[a]	References
	↓ability to clear viral (snakehead rhabovirus) or bacterial (*E. tarda*) load	Immune function (Zebrafish)	Zebrafish in vivo	2 or 10ppb sodium arsenite in water (4 days starting at 1 cell stage)	(Nayak et al., 2007)
	Respiratory infection ↑ influenza virus titer and ↑virus-related morbidity	Immune function (Mice)	Male C57bl/6j mice in vivo	100ppb sodium arsenite in drinking water (5 wks)	(Kozul et al., 2009)
↓contact sensitization response	↓Lymph node proliferation; ↓ear swelling to DNFB	Immune, Skin (Mice)	Female balb/c mice in vivo	50 mg/l sodium metaarsenite in drinking water (4 wks)	(Patterson et al., 2004)
Susceptible individual response					
NALP2 gene polymorphism (C/A +A/A) of NLP2 A1052E SNPs	NALP2 gene polymorphism modifies inorganic arsenic-associated respiratory disease	Immune / Respiratory (Human)	Individuals from West Bengal all with inorganic arsenic exposure; case-control study divided by presence of inorganic arsenic-related skin lesions	Exposure assessed by inorganic arsenic content of drinking water and urine samples	(Bhattacharjee et al., 2013)
TNFα and IL10 gene polymorphism (-308G/A and -3575T/A)	GA/AA TNFα genotype had higher risk of developing inorganic arsenic-induced conjunctivitis and respiratory effects; TNFα (pro-inflammatory cytokine) and IL10 (anti-inflammatory cytokine) gene polymorphisms modify serum TNFα and IL10 levels	Immune / Respiratory/ eye (Human)	Individuals from West Bengal all with inorganic arsenic exposure; case-control study divided by presence of inorganic arsenic-related skin lesions	Arsenic exposure assessed in urine	(Banerjee et al., 2011)
Population Response					
Respiratory infection	↑ relative risk of lower respiratory tract infection, ↑relative risk of sever lower respiratory tract infection	Immune/ Respiratory (Human)	Children in Matlab region of Bangladesh MINIM cohort	262–977 µg/L maternal arsenic metabolites in urine (average) compared to <39 µg/L maternal arsenic metabolites in urine	(Rahman et al., 2011)
	↑acute respiratory infection	Immune/ Respiratory (Human)	Children in Matlab region of Bangladesh cohort	152.4, 145.8 µg/L maternal arsenic metabolites in urine (mean) at weeks 8 and 30 gestation	(Raqib et al., 2009)

Key Events	Observations	Organ system	Test System	Dose (Exposure Duration)[a]	References
	↑ relative risk of lower and upper respiratory tract infection requiring physician visit or prescription medication, and ↑respiratory symptoms,	Immune/ Respiratory (Human)	Children New Hampshire Birth Cohort	6 µg/L maternal urinary As levels (mean) at 24–28 wks gestation	(Farzan et al., 2013)
Disease/cold fever	↑ days of fever	Immune (Human)	Pregnant mothers in Matlab region of Bangladesh cohort	152.4, 145.8 µg/L maternal arsenic metabolites in urine (mean) at weeks 8 and 30 gestation	(Raqib et al., 2009)
	↑colds treated with prescription	Immune (Human)	Children New Hampshire Birth Cohort	6 µg/L maternal urinary As levels(mean) at 24–28 wks gestation	(Farzan et al., 2013)
Infection-related GI disease	↑ relative risk of diarrhea	Immune/ gastro-intestinal (Human)	Children in Matlab region of Bangladesh MINIM cohort	262–977 µg/L maternal arsenic metabolites in urine (average) compared to <39 µg/L maternal arsenic metabolites in urine	(Rahman et al., 2011)
	↑ days of diarrhea	Immune/ gastro-intestinal (Human)	Pregnant mothers in Matlab region of Bangladesh cohort	152.4, 145.8 µg/L maternal arsenic metabolites in urine (mean) at weeks 8 and 30 gestation	(Raqib et al., 2009)
	↑diarrhea symptoms lasting two or more days or requiring doctors visit were associated but not significant [RR=1.9(0.9,3.9) and RR=3.5 (0.8,15.4)]	Immune/ Respiratory (Human)	Children New Hampshire Birth Cohort	6 µg/L maternal urinary As levels (mean) at 24–28 wks gestation	(Farzan et al., 2013)

[a]Abbreviations used for exposure durations: minutes (min), hrs (hr), days (d), weeks (wks).
[b]Exposure durations are characterized as "unspecified" when a study does not explicitly state the exposure duration.

Table A-6. Preliminary data on potential interactions between inorganic arsenic exposure and other chemicals or stressors

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
Susceptible Individuals					
Smoking	Multiple epidemiological studies have found smoking interacts with inorganic arsenic exposure to increase lung and bladder cancer risk	Lung Urinary bladder (Human)	Human Population	Variable	(Cohen et al., 2013) review
	Synergistic interaction of smoking and inorganic arsenic ingestion with skin lesions	Skin (Human)	Human population	Variable	(Chen et al., 2006); (Melkonian et al., 2011)
	Synergistic interaction between inorganic arsenic exposure and smoking in mortality from heart disease	Heart disease (Human)	Bangladesh	25.3–114 ppb	(Chen et al., 2011)
	Interaction between smoking and bladder-cancer risk (↑ odds ratio in ever smokers compared to never smokers; greater ↑ in odds ratio for smokers with shorter duration of As exposure compared to smokers with longer exposure duration)	Bladder (Human)	Human population (New Hampshire)	>0.330 µg/g toenail As conc. (Inorganic arsenic: 16.5 yrs [average]; Smoking: <15 yrs or ≤ 15 yrs)	(Karagas et al., 2004)
Co-exposures	Synergistic effects between fertilizer use and inorganic arsenic levels in drinking water for skin lesions; longer duration of fertilizer use associated with higher hazard ratio	Skin (Human)	Human population (Bangladesh)	>50 µg/L total arsenic in water (As: 10 yrs [mean]; Fertilizer: <10 yrs) >10 µg/L total arsenic in water (As: 10 yrs [mean]; Fertilizer: >10 yrs)	(Melkonian et al., 2011)
	Cd and As have cumulative effects on renal tubule leakage	Kidney	Humans	Mean concentration of Cd: 1.21.ppb and As: 5.7ppb	(Huang et al., 2009)
Diet	Low vegetable fiber, low calcium, low folate, and low animal protein may increase risk of skin lesions	Skin (Human)	Human Population (West Bengal, India)	<500 µg/L total arsenic (unspecified)	(Mitra et al., 2004)

Key events	Observations	Organ system	Test system	Dose (exposure duration)[a]	References
	Poor nutritional status (low body weight) associated with increased risk of skin lesions	Skin (Human)	Human Population (West Bengal, India)	<73.0 µg/kg/d total arsenic (unspecified)	(Guha Mazumder et al., 1998)
	Lower body-mass index associated with increased risk of skin lesions	Skin (Human)	Human Population (Bangladesh)	Variable	(Milton et al., 2004); (Ahsan et al., 2006)
	Lower dietary intake of folate and other B vitamins led to a stronger positive association between exposure and hypertension	Hypertension (Human)	Human population (Bangladesh)	<864 ppb	(Chen et al., 2007b)
	Development of skin lesions associated with low folate	Skin/Blood (human)	Peripheral blood lymphocyte DNA in individuals with skin lesions	2–250 µg/L As(III) (>2 yrs)	(Pilsner et al., 2009)
	Non-toxic inorganic arsenic exposure leads to enhanced inorganic arsenic accumulation when combined with Se-deficiency; could affect fetal brain development	Brain (Developing Mouse)	Pregnant ICR mice	58 µmol/kg/d sodium arsenite +/-Se-deficient diet	(Miyazaki et al., 2005)

APPENDIX B. ELECTRONIC DATABASE SEARCH STRATEGIES

Table B-1. Database search strategy

Date of search	Database	Search string
1/2013 12/2013 12/2016	PubMed	("arsenic"[MeSH Terms] OR "arsenic"[All Fields]) OR "7440-38-2"[All Fields] OR "inorganic arsenic"[All Fields] OR "monomethylarsenic"[All Fields] OR "dimethylarsenic"[All Fields] OR "methyl arsenic"[All Fields] OR "monomethylarsonic acid"[All Fields] OR (124[All Fields] AND 58[All Fields] AND 3[All Fields]) OR "monomethylarsonous acid"[All Fields] OR "dimethylarsinic acid"[All Fields] OR "75-60-5"[All Fields] OR "dimethylarsinous acid"[All Fields] OR "arsenate"[All Fields] OR (12523[All Fields] AND 21[All Fields] AND 6[All Fields]) OR "arsenite"[All Fields] OR (7784[All Fields] AND 46[All Fields] AND 5[All Fields]) OR "cacodylic acid"[All Fields] NOT "arsenic trioxide"[All Fields])
1/2013 12/2013 12/2016	Web of Science	(TS=arsenic OR TS="7440-38-2" OR TS="inorganic arsenic" OR TS=monomethylarsenic OR TS=dimethylarsenic OR TS=methylarsenic OR TS="monomethylarsonic acid" OR TS="124-58-3" OR TS="monomethylarsonous acid" OR TS="dimethylarsinic acid" OR TS="cacodylic acid" OR TS="75-60-5" OR TS="dimethylarsinous acid" OR TS=arsenate OR TS="12523-21-6" OR TS=arsenite OR TS="7784-46-5") NOT TS="arsenic trioxide" NOT WC="Geochemistry Geophysics" NOT WC="Physics Applied" NOT WC="Physics Condensed Matter" NOT WC="Materials Science Coatings Films" NOT WC=Optics NOT WC="Chemistry Physical" NOT WC=Mechanics NOT WC="Instruments Instrumentation" NOT WC="Engineering Manufacturing" NOT WC="Materials Science Characterization Testing" NOT WC=Electrochemistry NOT WC="Metallurgy Metallurgical Engineering" NOT WC="Chemistry Analytical" NOT WC="Engineering Environmental" NOT WC="Materials Science Multidisciplinary" NOT WC="Chemistry Inorganic Nuclear" NOT WC="Engineering Electrical Electronic" NOT WC="Engineering Chemical" NOT WC=Spectroscopy NOT WC=Crystallography NOT WC="Engineering Civil" NOT WC="Nanoscience Nanotechnology" NOT WC=Mineralogy NOT WC="Physics Atomic Molecular Chemical" NOT WC="Mining Mineral Processing" NOT WC="Energy Fuels" NOT WC="Materials Science Paper Wood" NOT WC="Materials Science Ceramics" NOT WC="Materials Science Characterization Testing" NOT WC="Physics Nuclear" NOT WC="Polymer Science" NOT WC=Geology NOT WC=Limnology NOT WC="Engineering Manufacturing" NOT WC="Agricultural Engineering" NOT WC="Engineering Mechanical" NOT WC="Computer Science Hardware Architecture" NOT WC="Imaging Science Photographic Technology")
1/2013 12/2013 12/2016	Toxline	(7440-38-2 OR 124-58-3 OR 75-60-5 OR 7784-46-5 OR arsenic OR "inorganic + arsenic" OR monomethylarsenic OR dimethylarsenic OR methylarsenic OR "monomethylarsonic acid" OR "monomethylarsonous acid" OR "dimethylarsinic acid" OR "dimethylarsinous acid" OR arsenate OR arsenite OR arsenicals) NOT "arsenic trioxide"

Note: Assessing the use of arsenicals, primarily arsenic trioxide and Fowler's solution, as chemotherapeutic agents are not within the scope to the review.

APPENDIX C. OFFICE OF HEALTH ASSESSMENT AND TRANSLATION (OHAT) (NTP, 2013) RISK-OF-BIAS QUESTIONS AND ASSESSMENT-SPECIFIC CLARIFICATIONS EXAMPLE

Table C-1 Risk of bias questions and rating guidelines—epidemiological studies

Rating	Guidelines and clarifications
1. Was administered dose or exposure level adequately randomized?	
++	**OHAT:** **Human-Controlled Trial**: There is direct evidence that subjects were allocated to any study group including controls using a method with a random component. Acceptable methods of randomization include referring to a random number table, using a computer random number generator, coin tossing, shuffling cards or envelopes, throwing dice, or drawing of lots (Higgins and Green, 2011). Restricted randomization (e.g., blocked randomization) to ensure allocation ratios will be considered low risk of bias. Similarly, stratified randomization and minimization approaches that attempt to minimize imbalance between groups on important prognostic factors (e.g., body weight) will be considered acceptable. **Assessment-Specific Clarification:** None.
+	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that subjects were allocated to study groups using a method with a random component (i.e., authors state that allocation was random, without description of the method used), OR it is deemed that allocation without a clearly random component during the study would not appreciably bias results. For example, approaches such as biased coin or urn randomization, replacement randomization, mixed randomization, and maximal randomization may require consultation with a statistician to determine risk-of-bias rating (Higgins and Green, 2011). **Assessment-Specific Clarification:** None.
–	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that subjects were allocated to study groups using a method with a nonrandom component, OR there is insufficient information provided about how subjects were allocated to study groups. Nonrandom allocation methods may be systematic but have the potential to allow participants or researchers to anticipate the allocation to study groups. Such "quasi-random" methods include alternation, assignment based on date of birth, case record number, or date of presentation to study (Higgins and Green, 2011). **Assessment-Specific Clarification:** None.
––	**OHAT:** **Human-Controlled Trial**: There is direct evidence that subjects were allocated to study groups using a nonrandom method including judgment of the clinician, preference of the participant, the results of a laboratory test or a series of tests, or availability of the intervention (Higgins and Green, 2011). **Assessment-Specific Clarification:** None.

Rating	Guidelines and clarifications
2. Was allocation to study groups adequately concealed?	
++	**OHAT:** **Human-Controlled Trial**: There is direct evidence that at the time of recruitment the research personnel and subjects did not know where study group subjects were allocated, and it is unlikely that they could have broken the blinding of allocation until after recruitment was complete and irrevocable. Methods used to ensure allocation concealment include central allocation (including telephone, web-based, and pharmacy-controlled randomization); sequentially numbered drug containers of identical appearance; sequentially numbered, opaque, sealed envelopes; or equivalent methods. **Assessment-Specific Clarification:** None.
+	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that the research personnel and subjects did not know where study group subjects were allocated, OR it is deemed that lack of adequate allocation concealment would not appreciably bias results. **Assessment-Specific Clarification:** None.
–	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that at the time of recruitment it was possible for the research personnel and subjects to know where study group subjects were allocated, or it is likely that they could have broken the blinding of allocation before recruitment was complete and irrevocable, OR there is insufficient information provided about allocation of study groups. Note: Inadequate methods include using an open random allocation schedule (e.g., a list of random numbers), assignment envelopes used without appropriate safeguards (e.g., if envelopes were unsealed or nonopaque or not sequentially numbered), alternation, or rotation; date of birth; case record number; or any other explicitly unconcealed procedure. For example, if the use of assignment envelopes is described, but it remains unclear whether envelopes were sequentially numbered, opaque, and sealed. **Assessment-Specific Clarification:** None.
– –	**OHAT:** **Human-Controlled Trial**: There is direct evidence that at the time of recruitment it was possible for the research personnel and subjects to know where study group subjects were allocated, or it is likely that they could have broken the blinding of allocation before recruitment was complete and irrevocable. **Assessment-Specific Clarification:** None.

Rating	Guidelines and clarifications
3. Were the comparison groups appropriate?	
++	**OHAT:** **Cohort, Cross-Sectional**: There is direct evidence that subjects (both exposed and nonexposed) were similar (e.g., recruited from the same eligible population, recruited with the same method of ascertainment using the same inclusion and exclusion criteria, and were of similar age and health status), recruited within the same time frame, and had the similar participation/response rates. **Case Control**: There is direct evidence that cases and controls were similar (e.g., recruited from the same eligible population including being of similar age, gender, ethnicity, and eligibility criteria other than outcome of interest as appropriate), recruited within the same time frame, and controls are described as having no history of the outcome. Note: A study will be considered low risk of bias if baseline characteristics of groups differed, but these differences were considered as potential confounding or stratification variables (see Question 4). **Assessment-Specific Clarification:** **Ecological and Semi-individual**: For ecological studies, a table of information or text on potential differences in characteristics that could bias results is provided, and these characteristics are adjusted for as potential confounders. There is direct evidence that subjects (both exposure groups and referent groups) were similar (e.g., of similar geographic region, ethnicity, socioeconomic status, etc.), OR baseline characteristics of groups differed but these differences were considered as potential confounding or stratification variables in analyses (see Question 4). **Additional Guidance:** Comparison groups selected adequately. Study provides table of subject characteristics by exposure levels and/or by case status. Cross-sectional studies can be considered low risk of bias if a general table of subject characteristics is provided and analyses are adjusted for confounders.
+	**OHAT:** **Cohort, Cross-Sectional**: There is indirect evidence that subjects (both exposed and nonexposed) were similar (e.g., recruited from the same eligible population, recruited with the same method of ascertainment using the same inclusion and exclusion criteria, and were of similar age and health status), recruited within the same time frame, and had the similar participation/response rates, OR differences between groups would not appreciably bias results. **Case Control**: There is indirect evidence that cases and controls were similar (e.g., recruited from the same eligible population, recruited with the same method of ascertainment using the same inclusion and exclusion criteria, and were of similar age), recruited within the same time frame, and controls are described as having no history of the outcome, OR differences between cases and controls would not appreciably bias results. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that subjects (both exposure groups and referent groups) were similar (e.g., of similar geographic region, ethnicity, socioeconomic status), OR differences between groups would not appreciably bias results. **Additional Guidance:** Recruitment methods stated to be similar, but no table of information or text provided on potential differences in study subjects' characteristics that could bias results, OR no breakdown of subject characteristics by exposure group (or by case status) to display potential differences. For ecological studies, groups are stated to be similar, but no table of information or text is provided on potential characteristic differences that could bias results.
–	**OHAT:** **Cohort, Cross-Sectional**: There is indirect evidence that subjects (both exposed and nonexposed) were not similar, recruited within very different time frames, or had very different participation/response rates, OR there is insufficient information provided about the comparison group including a different rate of nonresponse without an explanation. **Case Control**: There is direct evidence that controls were drawn from a very dissimilar population than cases or recruited within very different time frames, OR there is insufficient information provided about the appropriateness of controls including rate of response reported for cases only. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that subjects (both exposure groups and referent groups) were not similar (e.g., of similar geographic region, ethnicity, socioeconomic status), OR there is insufficient information provided about the appropriateness of comparison groups.

Rating	Guidelines and clarifications
– –	**OHAT:** **Cohort, Cross-Sectional**: There is direct evidence that subjects (both exposed and nonexposed) were not similar, recruited within very different time frames, or had very different participation/response rates. **Case Control**: There is direct evidence that controls were drawn from a very dissimilar population than cases or recruited within very different time frames. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that subjects (both exposure groups and referent groups) were not similar (e.g., of similar geographic region, ethnicity, socioeconomic status). **Additional Guidance:** At least one known difference between the groups was not accounted for (e.g., the study authors acknowledged that the groups were different with respect to a variable that is a potential confounder not considered in the analysis), OR recruitment methods were very different (e.g., recruitment completed during different time frames, different criteria were used for recruitment).
4. Did the study design or analysis account for important confounding and modifying variables?	
++	**OHAT:** **Human-Controlled Trial, Cohort, Cross-Sectional, Case Series/Report**: There is direct evidence that appropriate adjustments or explicit considerations were made for primary covariates and confounders in the final analyses through statistical models to reduce research-specific bias including standardization, case matching, adjustment in multivariate model, stratification, propensity scoring, or other methods were appropriately justified. Acceptable consideration of appropriate adjustment factors includes cases when the factor is not included in the final adjustment model because the author conducted analyses that indicated it did not need to be included. **Case Control**: There is direct evidence that appropriate adjustments were made for primary covariates and confounders in the final analyses through statistical models to reduce research specific bias including standardization, matching of cases and controls, adjustment in multivariate model, stratification, propensity scoring, or other methods were appropriately justified. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that appropriate adjustments or explicit considerations were made for covariates and confounders in the final analyses through statistical models (e.g., standardization, multivariate adjustment). Acceptable consideration of appropriate adjustment factors includes cases when the factor is not included in the final adjustment model because the author conducted analyses that indicated it did not need to be included. **Additional Guidance:** Study adjusted for or addressed important potential confounders. Age, gender, education, and socioeconomic status are potential confounders that need to be addressed and considered in the study design or analyses. In addition, specific important confounders for this assessment depend on the health outcome and include smoking for lung cancer, sun exposure for skin lesions, and alcohol drinking for hepatic outcomes. Other confounders might also be judged important for certain health outcomes. A low risk-of-bias rating was assigned for this question if potential confounders deemed important were adequately addressed (e.g., distribution of variables was compared between groups, and there was no statistically significant difference).
+	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is indirect evidence that appropriate adjustments were made for most primary covariates and confounders, OR it is deemed that not considering or only considering a partial list of covariates or confounders in the final analyses would not appreciably bias results. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that appropriate adjustments were made for most covariates and confounders, OR it is deemed that not considering or only considering a partial list of covariates or confounders in the final analyses would not appreciably bias results. **Additional Guidance:** Study adjusted only for some important potential confounders (e.g., sex and age), but it is likely that other confounders were present and not addressed (i.e., minimal number of confounders addressed).

Rating	Guidelines and clarifications
–	**OHAT:** **Human-Controlled Trial, Cohort, Cross-Sectional, Case Series/Report**: There is indirect evidence that the distribution of primary covariates and known confounders differed between the groups and was not appropriately adjusted for in the final analyses, OR there is insufficient information provided about the distribution of known confounders. **Case Control**: There is indirect evidence that the distribution of primary covariates and known confounders differed between cases and controls and was not investigated further, OR there is insufficient information provided about the distribution of known confounders in cases and controls. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that the distribution of covariates and known confounders differed between the groups and was not appropriately adjusted for in the final analyses, OR there is insufficient information provided about the distribution of known confounders. **Additional Guidance:** Design or analysis did not adjust for important potential confounders. Adjustments were made for some potential confounders, but at least one major confounder was not addressed (e.g., no adjustment for smoking when evaluating lung cancer, no adjustment for sun exposure when evaluating skin cancer).
– –	**OHAT:** **Human-Controlled Trial, Cohort, Cross-Sectional, Case Series/Report**: There is direct evidence that the distribution of primary covariates and known confounders differed between the groups, confounding was demonstrated, and was not appropriately adjusted for in the final analyses. **Case Control**: There is direct evidence that the distribution of primary covariates and known confounders differed between cases and controls, confounding was demonstrated, but was not appropriately adjusted for in the final analyses. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT Human-Controlled Trial, Cohort, Cross-Sectional, and Case Series/Report criteria. **Additional Guidance:** None.
5. Did researchers adjust or control for other exposures that are anticipated to bias results?	
++	**OHAT:** **Human-Controlled Trial**: There is direct evidence that other exposures anticipated to bias results were not present or were appropriately adjusted for. **Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence that other exposures anticipated to bias results were not present or were appropriately adjusted for. For occupational studies or studies of contaminated sites, other chemical exposures known to be associated with those settings were appropriately considered. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT Human-Controlled Trial criteria. **Additional Guidance:** Researchers adjusted for other chemicals or accounted for occupational exposures likely to be associated with the outcome.
+	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is indirect evidence that other coexposures anticipated to bias results were not present or were appropriately adjusted for, OR it is deemed that coexposures present would not appreciably bias results. Note, as discussed above, this includes insufficient information provided on coexposures in general population studies. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** No evidence that coexposures were addressed as confounders, but other specific chemicals or occupational exposures were addressed.

Rating	Guidelines and clarifications
–	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that the control group may have received the treatment or there was an unbalanced provision of additional coexposures, which were not appropriately adjusted for. **Cohort, Cross-Sectional, Case Series/Report**: There is indirect evidence that there was an unbalanced provision of additional coexposures across the primary study groups, which were not appropriately adjusted for, OR there is insufficient information provided about coexposures in occupational studies or studies of contaminated sites where high exposures to other chemical exposures would have been reasonably anticipated. **Case Control**: There is indirect evidence that there was an unbalanced provision of additional coexposures across cases and controls, which were not appropriately adjusted for, OR there is insufficient information provided about coexposures in occupational studies or studies of contaminated sites where high exposures to other chemical exposures would have been reasonably anticipated. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that there was an unbalanced provision of additional coexposures, which were not appropriately adjusted for, OR there is insufficient information provided about coexposures in studies of contaminated sites where high exposures to other chemical exposures would have been reasonably anticipated. **Additional Guidance:** There is evidence that coexposures might not have been addressed. Examples include a study population with farmers and/or other types of workers but occupational coexposures (e.g., to pesticides) not addressed; or a study with known coexposures, but the relevance of the coexposure to arsenic effects is unknown, or it is not clear if other compounds were adjusted for in the analyses.
– –	**OHAT:** **Human-Controlled Trial**: There is direct evidence that the control group received the treatment or there was an unbalanced provision of additional coexposures, which were not appropriately adjusted for. **Cohort, Cross-Sectional, Case Series/Report**: There is direct evidence that there was an unbalanced provision of additional coexposures across the primary study groups, which were not appropriately adjusted for. **Case Control**: There is direct evidence that there was an unbalanced provision of additional coexposures across cases and controls, which were not appropriately adjusted for. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that there was an unbalanced provision of additional coexposures, which were not appropriately adjusted for. **Additional Guidance:** Known differential exposure to other chemical/pollutant also associated with the health outcome of interest occurred with arsenic, and exposure was not addressed by the study authors. An example is a study of copper smelter workers where the study authors either (a) list other chemicals likely to be associated with the health outcome that the subjects were exposed to, or (b) provide levels of the other compounds, **AND** there were statistically significant differences related to the arsenic exposure that were not addressed. Such differences might have resulted from differential exposure to another compound or arsenic; thus, it cannot be determined which exposure impacted the results.
6. Were experimental conditions identical across study groups?	
NA	NA
7. Did researchers adhere to the protocol?	
++	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence that there were no deviations from the protocol (i.e., the study report explicitly provides this level of detail). **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** None.

Rating	Guidelines and clarifications
+	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is indirect evidence that there were no deviations from the protocol (i.e., authors did not report any deviations), OR deviations from the protocol are described and it is deemed that they would not appreciably bias results. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** Taking into consideration typical reporting practices, it seems unlikely that deviations from the protocol will be explicitly reported in most studies. Thus, unless stated otherwise by the authors (i.e., evidence of deviation is reported), or it is clear from the study report that deviations from the planned approach occurred, assume that no deviations occurred. It is anticipated that this approach will result in a rating of "probably low risk of bias" (+) for most studies. If there are deviations, the rating reflects how the deviations changed direction, magnitude, and/or significance of the results.
–	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/report**: There is indirect evidence that there were large deviations from the protocol as outlined in the methods or study report. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** None.
––	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence that there were large deviations from the protocol as outlined in the methods or study report. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** None.
8. Were the research personnel and human subjects blinded to the study group during the study?	
++	**OHAT:** **Human-Controlled Trial**: There is direct evidence that the subjects and research personnel were adequately blinded to study group, and it is unlikely that they could have broken the blinding during the study. Methods used to ensure blinding include central allocation, sequentially numbered drug containers of identical appearance; sequentially numbered, opaque, sealed envelopes; or equivalent methods. **Assessment-Specific Clarification:** None.
+	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that the research personnel and subjects were adequately blinded to study group, and it is unlikely that they could have broken the blinding during the study, OR it is deemed that lack of adequate blinding during the study would not appreciably bias results. **Assessment-Specific Clarification:** None.
–	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that it was possible for research personnel or subjects to infer the study group, OR there is insufficient information provided about blinding of the study group. Inadequate methods include using an open random allocation schedule (e.g., a list of random numbers), assignment envelopes used without appropriate safeguards (e.g., if envelopes were unsealed or nonopaque or not sequentially numbered), alternation, or rotation; date of birth; case record number; or any other explicitly unconcealed procedure. For example, if the use of assignment envelopes is described, but it remains unclear whether envelopes were sequentially numbered, opaque, and sealed. **Assessment-Specific Clarification:** None.

Rating	Guidelines and clarifications
−−	**OHAT:** Human-Controlled Trial: There is direct evidence for lack of adequate blinding of the study group including no blinding or incomplete blinding of research personnel and subjects. For some treatments, such as behavioral interventions, allocation to study groups cannot be concealed. **Assessment-Specific Clarification:** None.
9. Were outcome data complete without attrition or exclusion from analysis?	
++	**OHAT:** **Human-Controlled Trial**: There is direct evidence that there was no loss of subjects during the study and outcome data were complete, OR loss of subjects (i.e., incomplete outcome data) was adequately addressed and reasons were documented when human subjects were removed from a study. Review authors should be confident that the participants included in the analysis are exactly those who were randomized into the trial. Acceptable handling of subject attrition includes: very little missing outcome data, less than 10% in each group; (Genaidy et al., 2007); reasons for missing subjects unlikely to be related to outcome (for survival data, censoring unlikely to be introducing bias); missing outcome data balanced in numbers across study groups, with similar reasons for missing data across groups, OR analyses (such as intention-to-treat analysis) in which missing data have been imputed using appropriate methods (ensuring that the characteristics of subjects lost to follow up or with unavailable records are described in an identical way and are not significantly different from those of the study participants). Note: Participants randomized but subsequently found not to be eligible need not always be considered as having missing outcome data (Higgins and Green, 2011). **Cohort**: There is direct evidence that loss of subjects (i.e., incomplete outcome data) was adequately addressed and reasons were documented when human subjects were removed from a study. Acceptable handling of subject attrition includes: very little missing outcome data; reasons for missing subjects unlikely to be related to outcome (for survival data, censoring unlikely to be introducing bias); missing outcome data balanced in numbers across study groups, with similar reasons for missing data across groups; OR missing data have been imputed using appropriate methods, **AND** characteristics of subjects lost to follow up or with unavailable records are described in an identical way and are not significantly different from those of the study participants. **Case Control, Cross-Sectional**: There is direct evidence that exclusion of subjects from analyses was adequately addressed, and reasons were documented when subjects were removed from the study or excluded from analyses. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that there was no loss of subjects (e.g., due to moving or migration) or data during the study and outcome data were complete, OR incomplete outcome data were adequately addressed, **AND** characteristics of subjects lost to follow up or with unavailable records are described in an identical way and are not significantly different from those of the study participants. **Additional Guidance:** There are no reported data lost to attrition, and the numbers in the results tables sum to the total number of subjects, OR less than 10% of data are missing, OR there are some missing outcome data but study report clearly identifies missing data and how it was handled (e.g., loss to follow-up for a cohort study is determined to be minimal if there are some missing data for either the exposure or outcome for certain subjects at a specific time measured and the authors clearly explain what happened to everyone and which results were used in the analyses). For ecological studies specifically, there are no reported data lost to attrition, OR there are some missing data but study report clearly identifies missing data and how they were handled (e.g., migration in and out of study area and residence location within study area were tracked and accounted for or references provided to verify that population migration within or in/out of study area is not a concern for this population), and characteristics of subjects lost to attrition do not differ significantly from those included in study.

Rating	Guidelines and clarifications
+	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that loss of subjects (i.e., incomplete outcome data) was adequately addressed and reasons were documented when human subjects were removed from a study, OR it is deemed that the proportion lost to follow-up would not appreciably bias results, less than 20% in each group; (Genaidy et al., 2007). This would include reports of no statistical differences in characteristics of subjects lost to follow up or with unavailable records from those of the study participants. Generally, the higher the ratio of participants with missing data to participants with events, the greater potential there is for bias. For studies with a long duration of follow-up, some withdrawals for such reasons are inevitable. **Cohort**: There is indirect evidence that loss of subjects (i.e., incomplete outcome data) was adequately addressed and reasons were documented when human subjects were removed from a study, OR it is deemed that the proportion lost to follow-up would not appreciably bias results. This would include reports of no statistical differences in characteristics of subjects lost to follow-up or with unavailable records from those of the study participants. Generally, the higher the ratio of participants with missing data to participants with events, the greater potential there is for bias. For studies with a long duration of follow-up, some withdrawals for such reasons are inevitable. **Case Control, Cross-Sectional**: There is indirect evidence that exclusion of subjects from analyses was adequately addressed, and reasons were documented when subjects were removed from the study or excluded from analyses. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that there was no loss of subjects (e.g., due to migration during the study) and outcome data were complete, OR it is deemed that the proportion of subjects lost to follow-up would not appreciably bias results. This would include reports of no statistical differences in characteristics of subjects lost to follow-up or with unavailable records of outcomes. For studies with a long duration of follow-up, some withdrawals for such reasons are inevitable. **Additional Guidance:** No direct evidence of loss to follow-up, attrition, or loss of subjects due to migration/moving provided. The tables of results do not include the number of subjects and it is not stated that there was any loss data missing; OR there appear to be no or very few missing data; OR in a cohort study, there is no mention of loss to follow-up.
–	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that loss of subjects (i.e., incomplete outcome data) was unacceptably large [greater than 20% in each group; (Genaidy et al., 2007) and not adequately addressed, OR there is insufficient information provided about numbers of subjects lost to follow-up. **Cohort**: There is indirect evidence that loss of subjects (i.e., incomplete outcome data) was unacceptably large and not adequately addressed, OR there is insufficient information provided about numbers of subjects lost to follow-up. **Case Control, Cross-Sectional**: There is indirect evidence that exclusion of subjects from analyses was not adequately addressed, OR there is insufficient information provided about why subjects were removed from the study or excluded from analyses. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that incomplete outcome data (e.g., due to subject migration or moving) were unacceptably large [greater than 20% in each group; (Genaidy et al., 2007) and not adequately addressed, OR there is insufficient information provided about missing outcome data. **Additional Guidance:** Missing outcome data with no explanation of why data were missing, and it is unclear from the characteristics table or other information provided in the report why the data might be missing.

Rating	Guidelines and clarifications
– –	**OHAT:** **Human-Controlled Trial, Cohort**: There is direct evidence that loss of subjects (i.e., incomplete outcome data) was unacceptably large and not adequately addressed. Unacceptable handling of subject attrition includes reason for missing outcome data likely to be related to true outcome, with either imbalance in numbers or reasons for missing data across study groups, or potentially inappropriate application of imputation. **Case Control, Cross-Sectional**: There is direct evidence that exclusion of subjects from analyses was not adequately addressed. Unacceptable handling of subject exclusion from analyses includes reason for exclusion likely to be related to true outcome, with either imbalance in numbers or reasons for exclusion across study groups. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that incomplete outcome data were unacceptably large and not adequately addressed, OR that characteristics of subjects lost to attrition were significantly different from those included in study. **Additional Guidance:** The missing outcome data are clearly related to exposure (more missing data for exposed compared to unexposed groups), but the study authors do not address why. For ecological studies, there is unacceptable handling of subject migration into and out of the study area or subject residence locations within study area.
10. Were the outcome assessors blinded to study group or exposure level?	
++	**OHAT:** **Human-Controlled Trial**: There is direct evidence that the outcome assessors (including study subjects, if outcomes were self-reported) were adequately blinded to the study group, and it is unlikely that they could have broken the blinding prior to reporting outcomes. **Cohort, Cross-Sectional, Case Series/Report**: There is direct evidence that the outcome assessors (including study subjects, if outcomes were self-reported) were adequately blinded to the exposure level, and it is unlikely that they could have broken the blinding prior to reporting outcomes. **Case Control**: There is direct evidence that the outcome assessors (including study subjects if outcomes were self-reported) were adequately blinded to the exposure level when reporting outcomes. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT Cohort, Cross-Sectional, and Case Series/Report criteria. **Additional Guidance:** The study report states that outcome assessors were blinded to subjects' exposure levels, OR in a case-control study, researchers who assigned exposure levels based on drinking water level were blinded to the case/control status of the participant.
+	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that the outcome assessors (including study subjects, if outcomes were self-reported) were adequately blinded to the study group, and it is unlikely that they could have broken the blinding prior to reporting outcomes, OR it is deemed that lack of adequate blinding of outcome assessors would not appreciably bias results, which may vary by outcome (i.e., blinding is especially important for subjective measures). **Cohort, Cross-Sectional, Case Series/Report**: There is indirect evidence that the outcome assessors were adequately blinded to the exposure level, and it is unlikely that they could have broken the blinding prior to reporting outcomes, OR it is deemed that lack of adequate blinding of outcome assessors would not appreciably bias results (including that subjects self-reporting outcomes were likely not aware of reported links between the exposure and outcome lack of blinding is unlikely to bias a particular outcome). **Case Control**: There is direct evidence that the outcome assessors were adequately blinded to the exposure level when reporting outcomes, OR it is deemed that lack of adequate blinding of outcome assessors would not appreciably bias results (including that subject's self-reporting outcomes were likely not aware of reported links between the exposure and outcome or lack of blinding is unlikely to bias a particular outcome). **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT Human-Controlled Trial criteria. **Additional Guidance:** No direct statement that outcome assessors were blind, but it is likely that they were (e.g., pathologists conducting histopathology on the tissue would most likely be blind to the exposure status), OR outcomes were assessed using an automated instrument, making it unlikely that the results would be biased because automated instrument would not be biased.

Rating	Guidelines and clarifications
–	**OHAT:** **Human-Controlled Trial**: There is indirect evidence that it was possible for outcome assessors (including study subjects if outcomes were self-reported) to infer the study group prior to reporting outcomes, OR there is insufficient information provided about blinding of outcome assessors. **Cohort, Cross-Sectional, Case Series/Report**: There is indirect evidence that it was possible for outcome assessors to infer the exposure level prior to reporting outcomes (including that subject's self-reporting outcomes were likely aware of reported links between the exposure and outcome), OR there is insufficient information provided about blinding of outcome assessors. **Case Control**: There is indirect evidence that it was possible for outcome assessors to infer the exposure level prior to reporting outcomes (including that subject's self-reporting outcomes were likely aware of reported links between the exposure and outcome), OR there is insufficient information provided about blinding of outcome assessors. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT Case-Control criteria. **Additional Guidance:** Not enough information to determine if outcome assessors were blind to exposure status and the possibility exists that they could have knowledge (e.g., it is a cohort and exposure was assessed prior to outcome), OR likely that outcome assessors were aware of exposure, but not necessarily level of exposure (e.g., outcome was assessed in subject's home, which is in either the control village or exposed village, but the study report evaluated different exposure levels in village so that when assessing the outcome, assessors would be aware that subjects were exposed or controls but not exact exposure level).
– –	**OHAT:** **Human-Controlled Trial**: There is direct evidence for lack of adequate blinding of outcome assessors (including study subjects if outcomes were self-reported), including no blinding or incomplete blinding. **Cohort, Cross-Sectional, Case Series/Report**: There is direct evidence that outcome assessors were aware of the exposure level prior to reporting outcomes (including that subject's self-reporting outcomes were aware of reported links between the exposure and outcome). **Case Control**: There is direct evidence that outcome assessors were aware of the exposure level prior to reporting outcomes (including that subject's self-reporting outcomes were aware of reported links between the exposure and outcome). **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT Case-Control criteria. **Additional Guidance:** There is direct evidence that outcome assessor knew exposure status (e.g., same situation as above with outcome assessed in the village, but the report only evaluates exposure as "exposed vs. unexposed," with no arsenic levels measured).
11. Were confounding variables assessed consistently across groups using valid and reliable measures?	
++	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence that primary covariates and confounders were assessed using valid and reliable measurements. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that group- or individual-level primary covariates and confounders were assessed using valid and reliable measurements. **Additional Guidance:** Methods provide specific details on how confounders were measured (e.g., for body weight, details provided to indicate precision of measurement instrument and, ideally, calibration of instrument). Validated or pretested questionnaires used, and there was low potential for interviewer bias.

Rating	Guidelines and clarifications
+	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is indirect evidence primary covariates and confounders were assessed using valid and reliable measurements, OR it is deemed that the measures used would not appreciably bias results (i.e., the authors justified the validity of the measures from previously published research). **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that group- or individual-level primary covariates and confounders were assessed using valid and reliable measurements, OR it is deemed that the measures used would not appreciably bias results (i.e., the authors justified the validity of the measures from previously published research). **Additional Guidance:** Self-administered questionnaire, OR questionnaire administered by a single interviewer for all subjects (thus eliminating the possibility for interviewer agreement bias), OR methods for assessing confounders were mixed (e.g., some methods well conducted and consistent, but others may have been obtained from questionnaires not stated to be validated).
–	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is indirect evidence that primary covariates and confounders were assessed using measurements of unknown validity, OR there is insufficient information provided about the measures used. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that group- and individual-level primary covariates and confounders were assessed using measurements of unknown validity, OR there is insufficient information provided about the measures used. **Additional Guidance:** Not enough details were provided on how the confounders were assessed. Questionnaire used and administered by several interviewers with no details on validity/reliability of the questionnaire or on consistency between the interviewers.
– –	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence that primary covariates and confounders were assessed using nonvalid measurements. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that group- or individual-level primary covariates and confounders were not assessed using valid and reliable measures. **Additional Guidance:** There is direct evidence of selective recall by disease status.

Rating	Guidelines and clarifications
12. Can we be confident in the exposure characterization?	
++	**OHAT:** **Human-Controlled Trial**: There is direct or indirect evidence that the test material is confirmed as ≥99% pure (or impurities have been characterized and not considered to be of serious concern), and that the concentration, stability, and homogeneity of stock material and formulation have been verified as appropriate (Note: ≥99% purity value is considered achievable based on current advertised purity from Sigma-Aldrich); **AND FOR INTERNAL DOSIMETRY STUDIES** there is direct evidence that most data points for the aglycone, conjugated and/or total bisphenol A (BPA) are *above* the LOQ for the assay; **AND** the study used spiked samples to confirm assay performance and the stability of BPA and conjugated BPA in biological samples was appropriately addressed; **AND** studies took measures to assess potential BPA contamination that might have occurred during sample collection and analysis, including method blanks. Note: Use of method blanks is necessary to identify potential sources of contamination in blood and urine but cannot rule out all possible sources of contamination (Ye et al., 2012). The risk of contamination for blood-based measurements is likely higher than for urinary measurements in part because sterile plastic blood collection containers can increase the number of sources of contamination and because of higher levels of protein and lipid levels in blood vs. urine. Preferred practices include (1) measurement of aglycone and conjugated or total BPA for blood measurements, and (2) use of isotopically labeled BPA dosing material (e.g., deuterated) to avoid issues of contamination, although we will not "downgrade" if a study did not follow these preferred practices. **Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence that most data points for the aglycone, conjugated and/or total BPA are *above* the LOQ for the assay; **AND** the study used spiked samples to confirm assay performance and the stability of BPA and conjugated BPA in biological samples was appropriately addressed; **AND** studies took measures to assess potential BPA contamination that might have occurred during sample collection and analysis including method blanks. Note: Use of method blanks is necessary to identify potential sources of contamination in blood and urine but cannot rule out all possible sources of contamination (Ye et al., 2012). The risk of contamination for blood-based measurements is likely higher than for urinary measurements in part because sterile plastic blood collection containers can increase the number of sources of contamination and because of higher levels of protein and lipid levels in blood vs. urine. Preferred practices include (1) measurement of aglycone and conjugated or total BPA for blood measurements, and (2) inclusion of multiple measurements of BPA because a single sample from an individual does not appear to be strong predictor of a subject's exposure category. (Mahalingaiah et al., 2008) analyzed samples from at least six repeat urinary BPA measurements from eight subjects. The sensitivity, specificity, and positive predictive value of a single urine sample to predict the highest BPA tertiles were 0.64, 0.76, and 0.63, respectively. The positive predictive value increased to 0.85 when two samples were used to predict those individuals in the highest BPA tertile. Use of a single measurement in large sample size studies such as NHANES is less of an issue because the number of participants offsets potential concern for differential exposure misclassification. We will not downgrade if a study did not follow these preferred practices. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: This rating is not applicable. Only studies with individual-level exposure characterization can earn this rating. If individual-level exposure data are provided, the study is not an ecological study, and should be reclassified and rated according to other study type ROB criteria. **Additional Guidance:** Single spot urine samples are reported for many subjects (over 1,000), OR multiple (repeated) spot urine samples were reported. Individual-level drinking water levels (e.g., obtained from household tap or household well, but not village-level well) with methods well described, including reporting of LODs. Toenail and hair samples were cleaned, **AND** the recovery rate of the method or use of internal standards is reported. More than one arsenic exposure assessment (more than one matrix, and/or more than one measurement), and at least one of them is excellent (e.g., the large HEALS cohort and spot urine spot samples, in addition to village-level water arsenic measurements) and a correlation reported between the different measurements.

Rating	Guidelines and clarifications
+	**OHAT:** **Human-Controlled Trial**: There is direct or indirect evidence that purity was ≥98%, (or impurities have been characterized and not considered to be of serious concern, i.e., purity was independently confirmed by lab, purity is reported in paper or obtained through author query, or purity not reported but the source is listed and the supplier of the chemical provides documentation of the purity of the chemical; **AND FOR INTERNAL DOSIMETRY STUDIES**, there is indirect evidence that most data points for the aglycone, conjugated and/or total BPA are *above* the LOQ for the assay, i.e., the central estimate (median, mean, geometric mean) is *above* the LOQ but results for individual data values are not presented or the presentation of variance estimates does not permit assessment of whether most data points are likely *above* the LOQ; **AND** the study used spiked samples to confirm assay performance and the stability of BPA and conjugated BPA in biological samples was appropriately addressed; **AND** studies took measures to assess potential BPA contamination that might have occurred during sample collection and analysis including method blanks. **Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is indirect evidence that most data points for the aglycone, conjugated and/or total BPA are *above* the LOQ for the assay, i.e., the central estimate (median, mean, geometric mean) is *above* the LOQ but results for individual data values are not presented or the presentation of variance estimates do not permit assessment of whether most data points are likely *above* the LOQ; **AND** the study used spiked samples to confirm assay performance and the stability of BPA and conjugated BPA in biological samples has been appropriately addressed; **AND** studies took measures to assess potential BPA contamination that might have occurred during sample collection and analysis including method blanks; OR use of questionnaire items where results of biomonitoring studies support the use of the questionnaire item(s) as an indicator of relative level of exposure; OR job description for occupational studies where levels in the work environment or results of biomonitoring studies support the use of job description as an indicator of relative level of exposure. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct or indirect evidence that the exposure to the chemical of concern was adequately characterized by appropriate measures and methods (e.g., adequate monitoring over time of multiple sources per exposure group, cumulative exposures based on historical changes in measured exposures, exposure measures taken for a moderate proportion of population). **Additional Guidance:** Single spot urine samples with a moderate number of subjects (i.e., hundreds or more). Adequate measurements and methods, but limits of detection (LOD) are not provided. Exposure based on occupational title but supported by some arsenic monitoring (air, urine, or other biomarker). For ecological studies, drinking water levels were obtained from the smallest groups available (e.g., household or village level) with methods well described and monitoring over time to estimate cumulative exposure based on changes in arsenic concentrations, including reporting of LODs and residential durations.

Rating	Guidelines and clarifications
–	**OHAT:** **Human-Controlled Trial**: Neither the source nor purity of the chemical was reported in the study and information on purity could not be obtained through author query/vendor documentation; **AND FOR INTERNAL DOSIMETRY STUDIES**, there is direct or indirect evidence that most data points for the aglycone, conjugated, and/or total BPA are above the LOQ for the assay, BUT no steps were taken to assess potential BPA contamination that might have occurred during sample collection and analysis; OR there is indirect or direct evidence that most individual data points for the aglycone, conjugated, and/or total BPA are below the LOQ for the assay; OR method to measure BPA used ELISA, which is less accepted as providing quantitatively accurate values and because of potential uncharacterized antibody cross-reactivity with conjugates and endogenous components of sample matrices (Chapin et al., 2008);(Vandenberg et al., 2007). **Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct or indirect evidence that most data points for the aglycone, conjugated, and/or total BPA are above the LOQ for the assay, BUT no steps were taken to assess potential BPA contamination that might have occurred during sample collection and analysis; OR there is indirect or direct evidence that most individual data points for the aglycone, conjugated, and/or total BPA are below the LOQ for the assay; OR method to measure BPA used ELISA, which leads to concern because of uncharacterized antibody cross-reactivity with conjugates and endogenous components of sample matrices (Chapin et al., 2008);(Vandenberg et al., 2007); OR use of questionnaire items that are not supported by results of biomonitoring studies; OR job description for occupational studies that are not supported by information on levels in the work environment or results of biomonitoring studies. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that the chemical in question was not adequately characterized by appropriate measures and methods (e.g., no historical monitoring, isolated or remote-time samples taken to be representative of large areas, no cumulative exposures estimated). **Additional Guidance:** Exposure based on single spot urine sample for a limited number of subjects (less than 100), OR exposure based on occupational title with no arsenic monitoring, OR cumulative arsenic levels based on self-reported duration/resident history and group well-water measurements.
– –	**OHAT:** **Human-Controlled Trial**: There is indirect or direct evidence that purity was <98%; **AND FOR INTERNAL DOSIMETRY STUDIES**, there is direct evidence of uncontrolled contamination. **Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence of uncontrolled contamination, OR not reporting of methods used to assess exposure and this information could not be obtained through author query, OR self-report exposure. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that the chemical in question was not adequately characterized by appropriate measures and methods (e.g., no historical monitoring, isolated or remote-time samples taken to be representative of large areas, no cumulative exposures estimated), OR there is direct evidence of uncontrolled contamination, OR methods used to assess exposure not reported, OR self-reported exposure. **Additional Guidance:** No measured arsenic concentrations. Exposure assessed based on presence/absence of skin lesions, OR self-reported duration of drinking water or living in a certain area, OR lifetime cumulative arsenic exposure determined using self-reported information on residential history and drinking-water daily consumption rates, and village-level median arsenic concentration in drinking water.

Rating	Guidelines and clarifications
13. Can we be confident in the outcome assessment?	
++	**OHAT:** **Human-Controlled Trial, Cohort**: There is direct evidence that the outcome was assessed using well-established methods, the "gold standard," or with validity and reliability >0.70 (Genaidy et al., 2007), and subjects had been followed for the same length of time in all study groups. Acceptable assessment methods will depend on the outcome, but examples of such methods may include objectively measured with diagnostic methods, measured by trained interviewers, obtained from registries (Shamliyan et al., 2010). **Case Control**: There is direct evidence that the outcome was assessed in cases using well-established methods (the gold standard) and subjects had been followed for the same length of time in all study groups. **Cross-Sectional, Case Series/Report**: There is direct evidence that the outcome was assessed using well-established methods (the gold standard). **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that the outcome was assessed using well-established methods, the gold standard (e.g., individual-level outcome data were assessed, as in the case of semi-individual ecological studies), and subjects have been followed for the same length of time in all study groups. Acceptable assessment methods will depend on the outcome, but examples of such methods may include: objectively measured with diagnostic methods, measured by trained interviewers, obtained from reliable registries or records. **Additional Guidance:** Cancer cases are histologically confirmed, OR data obtained from nationwide registry are accepted as valid and complete (e.g., Taiwan), OR outcome diagnosed by physician, OR outcome obtained from medical record data or validated with such data (if self-reported).
+	**OHAT:** **Human-Controlled Trial, Cohort**: There is indirect evidence that the outcome was assessed using acceptable methods [i.e., deemed valid and reliable but not the gold standard or with validity and reliability ≥0.40; (Genaidy et al., 2007) and subjects had been followed for the same length of time in all study groups, OR it is deemed that the outcome assessment methods used would not appreciably bias results. Acceptable, but not ideal assessment methods will depend on the outcome, but examples of such methods may include proxy reporting of outcomes and mining of data collected for other purposes. **Case Control**: There is indirect evidence that the outcome was assessed in cases (i.e., case definition) using acceptable methods and subjects had been followed for the same length of time in all study groups, OR it is deemed that the outcome assessment methods used would not appreciably bias results. **Cross-Sectional, Case Series/Report**: There is indirect evidence that the outcome was assessed using acceptable methods, OR it is deemed that the outcome assessment methods used would not appreciably bias results. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that the outcome was assessed using acceptable methods (i.e., deemed valid and reliable but not the gold standard) and subjects had been followed for the same length of time in all study groups, OR it is deemed that the outcome assessment methods used would not appreciably bias results, OR group-level outcomes were assessed using well-established methods. Acceptable, but not ideal assessment methods will depend on the outcome, but examples of such methods may include proxy reporting of outcomes and mining of data collected for other purposes. **Additional Guidance:** Death certificates are used, but there is no statement that they were coded by certified nosologist, OR information on the accuracy/validity/completeness of the death certificates is missing, OR incident cancer cases are not stated to be histologically confirmed, but the study was conducted in a hospital setting (e.g., hospital-based case-control study).

Rating	Guidelines and clarifications
–	**OHAT:** **Human-Controlled Trial, Cohort**: There is indirect evidence that the outcome assessment method is an insensitive instrument, the authors did not validate the methods used, or the length of follow up differed by study group, OR there is insufficient information provided about validation of outcome assessment method. **Case Control**: There is indirect evidence that the outcome was assessed in cases using an insensitive instrument or was not adequately validated, OR there is insufficient information provided about how cases were identified. **Cross-Sectional, Case Series/Report**: There is indirect evidence that the outcome assessment method is an insensitive instrument or was not adequately validated, OR there is insufficient information provided about validation of outcome assessment method. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is indirect evidence that the authors did not validate the methods used, or the length of follow-up differed by study group, OR there is insufficient information provided about validation of outcome assessment method. **Additional Guidance:** Outcome is self-reported (e.g., "ever been diagnosed by a physician") and not verified by medical records or other means. There is insufficient information on quality of self-report or validation of answers. Outcome is assessed by nurses and there is no information on assessor agreement.
– –	**OHAT:** **Human-Controlled Trial, Cohort**: There is direct evidence that the outcome assessment method is an insensitive instrument, or the length of follow-up differed by study group. **Case Control**: There is direct evidence that the outcome was assessed in cases using an insensitive instrument. **Cross-Sectional, Case Series/Report**: There is direct evidence that the outcome assessment method is an insensitive instrument. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: There is direct evidence that the authors did not validate the methods used, or the length of follow-up differed by study group. **Additional Guidance:** Self-reported outcome when question is not worded "as diagnosed by a physician" and cannot be verified.
14. Were all measured outcomes reported?	
++	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence that all the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported. This would include outcomes reported with sufficient detail to be included in meta-analysis or fully tabulated during data extraction. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** None.
+	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is indirect evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported, OR analyses that had not been planned at the outset of the study (i.e., retrospective unplanned subgroup analyses) are clearly indicated as such, and it is deemed that the omitted analyses were not appropriate and selective reporting would not appreciably bias results. This would include outcomes reported with insufficient detail such as only reporting that results were statistically significant (or not). **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** All outcomes outlined in abstract, introduction, and methods are reported.

Rating	Guidelines and clarifications
–	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is indirect evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported, OR there is insufficient information provided about selective outcome reporting. **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** An outcome mentioned in a part of the study report is obviously missing from the results.
– –	**OHAT:** **Human-Controlled Trial, Cohort, Case Control, Cross-Sectional, Case Series/Report**: There is direct evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have not been reported. In addition to not reporting outcomes, this would include reporting outcomes based on composite score without individual outcome components or outcomes reported using measurements, analysis methods or subsets of the data (e.g., subscales) that were not prespecified or reporting outcomes not prespecified (unless clear justification for their reporting is provided, such as an unexpected effect). **Assessment-Specific Clarification:** **Ecological and Semi-individual**: Same as OHAT criteria. **Additional Guidance:** None.
15. Were there no other potential threats to internal validity (e.g., statistical methods were appropriate)?	
	OHAT: On a project-specific basis, additional questions for other potential threats to internal validity can be added and applied to study designs as appropriate.
++	**Assessment-Specific Clarification:** Statistical analyses were appropriate and no other threats to internal validity were identified. Study authors might acknowledge limitations, but these are not expected to affect the study's internal validity.
+	**Assessment-Specific Clarification:** There are study limitations likely to bias the results toward or away from the null, but adequate sample size was available in each cell ($n \geq 5$), OR sample size is small and acknowledged as a potential limitation by study authors, but significant results were still observed.
–	**Assessment-Specific Clarification:** There are study limitations likely to bias results towards or away from the null, OR analyses were conducted on a small number of subjects ($n < 5$ in any given cell) and no statistically significant results were observed.
– –	**Assessment-Specific Clarification:** None.

LOD = limit of detection; LOQ = level of quantitation; OHAT = Office of Health Assessment and Translation.

APPENDIX D. TYPICAL DATA ABSTRACTION FIELDS

Table D-1. Key data extraction elements to summarize study design, methodology, and results

Data abstraction field	Data extraction elements
HUMAN	
Funding	Funding source(s)
	Reporting of conflict of interest by authors
Subjects	Study population name/description
	Dates of study and sampling time frame
	Geography (country, region, state, etc.)
	Demographics (sex, race/ethnicity, age, or life stage at exposure and at outcome assessment)
	Number of subjects (target, enrolled, *n* per group in analysis, and participation/follow-up rates)
	Inclusion/exclusion criteria/recruitment strategy
	Description of reference group
Methods	Study design (e.g., prospective, or retrospective cohort, nested case-control study, cross-sectional, population-based case-control study, intervention, case report, etc.)
	Length of follow-up
	Health outcome category (e.g., cardiovascular)
	Health outcome (e.g., blood pressure)
	Diagnostic or methods used to measure health outcome
	Confounders or modifying factors and how considered in analysis (e.g., included in final model, considered for inclusion but determined not needed)
	Chemical name and CAS number
	Exposure assessment (e.g., blood, urine, hair, air, drinking water, job classification, residence, administered treatment in controlled study, etc.)
	Methodological details for exposure assessment (e.g., HPLC-MS/MS, limit of detection)
	Statistical methods
Results	Exposure levels (e.g., mean, median, measures of variance as presented in paper, such as standard deviation [SD], SEM, 75^{th}/90^{th}/95^{th} percentile, minimum/maximum); range of exposure levels, number of exposed cases

Data abstraction field	Data extraction elements
	Statistical findings (e.g., adjusted β, standardized mean difference, adjusted odds ratio, standardized mortality ratio, relative risk, etc.) or description of qualitative results. When possible, convert measures of effect to a common metric with associated 95% confidence intervals. Most often, measures of effect for continuous data are expressed as mean difference, standardized mean difference, and percentage control response. Categorical data are typically expressed as odds ratio, relative risk (RR, also called risk ratio), or β values, depending on what metric is most reported in the included studies and ability to obtain information for effect conversions from the study or through author query.
	Observations on dose-response (e.g., trend analysis, description of whether dose-response shape appears to be monotonic, nonmonotonic)
Other	Documentation of author queries, use of digital rulers to estimate data values from figures, exposure unit, and statistical result conversions, etc.

HPLC MS/MS = high performance liquid chromatography with mass spectrometry detection; SEM = standard error of the mean.

APPENDIX E. EVALUATION OF PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS

E.1. Model Choice

This appendix evaluates previously published physiologically based pharmacokinetic (PBPK) models for arsenic (As) (see Table E-1). Computational code for all published models was requested from the respective authors but was obtained only for the rabbit and hamster model of (Mann et al., 1996a), the mouse and rat model of (Gentry et al., 2004), and two human models (Lee, 1999); (El-Masri and Kenyon, 2008). All these models were written in the acsl programming language, but in different software versions or for different platforms. The model code of (Mann et al., 1996a) was written in Simulsolv Version 2.1 (Dow Chemical Co.), and that of (Gentry et al., 2004) was adapted from (Mann et al., 1996a). The model of (Lee, 1999) was written in acsl Tox (no specifications related to the version were found in the thesis), and the one by (El-Masri and Kenyon, 2008) was written with acslX. Any model selected for use would need to be converted to a currently available platform. A combination of R (for model scripts) and MCSim (for the core model code) is currently being used for other PBPK models and would be the first choice for this assessment because both platforms are open source and freely available. (Liao et al., 2008) (human child model) contains an appendix with a relatively good description of the equations, but the code would need to be built based on these equations and tested to verify the results match those in the paper.

Since 1996 when Mann et al. created their hamster and rabbit PBPK model, marked improvements in the biological description of arsenic absorption, distribution, metabolism, and excretion (ADME) processes have occurred. (El-Masri and Kenyon, 2008) used newer and more relevant experimental data. The biological relevance of the parameters used in the newer models has also increased so that this appendix does not need to address changes in the way parameter optimization or fitting was conducted. The greater biological relevance increases the confidence in the most recent model (El-Masri and Kenyon, 2008).

Table E-1 Models code information[a]

References	Model code (software)	Comments
(Mann et al., 1996a)	Simulsolv Version 2.1 (Dow Chemical Co.)	Adaptation needed
(Lee, 1999)	acsl Tox (see the Appendix)	Adaptation needed
(Liao et al., 2008)	MATLAB from MathWorks	Adaptation needed
(Gentry et al., 2004)	Probably acsl, but an old version	Adaptation needed
(El-Masri and Kenyon, 2008)	acslX (recent version)	Ready to evaluate in acslX

[a]All models would require adaptation to R/MCSim or another currently available platform for use.

E.1.1. (El-Masri and Kenyon, 2008) (human model)

The (El-Masri and Kenyon, 2008) PBPK model for As was developed for human adults and incorporates all the different forms of As. These include As(III), As(V), monomethylarsonate (MMA[III]), MMA(V), dimethylarsinate (DMA[III]), and DMA[V], although MMA(III) and DMA(III) are only described in the liver, lung, and kidney, with urinary excretion of MMA(III) and DMA(III) treated as occurring directly from those tissues. This model has eight compartments with flow-limited distribution: lung, liver, gastrointestinal (GI) tract (lumen and tissue), kidney, muscle, brain, skin, and heart (see Figure E-1). The physiological parameters came from (Brown et al., 1997). As(III), As(V), MMA(V), and DMA(V) are distributed in the systemic circulation simultaneously. While the model has lung and skin compartments, it is only coded and parameterized for oral absorption. Oral absorption and urinary elimination are described as first-order processes. Partition coefficients (PCs) were estimated from (Benramdane et al., 1999) and (Saady et al., 1989) for each form of circulating As. Total As levels in blood compared with tissues, as reported by (Saady et al., 1989), were considered accurate. (Benramdane et al., 1999) reported the fraction as As(III), As(V), MMA, and DMA, but samples were taken 3 days postmortem and the blood: tissue ratios were not considered reliable. For this reason, (El-Masri and Kenyon, 2008) used the ratios of As species from (Benramdane et al., 1999) together with the total blood: tissue ratios from (Saady et al., 1989) to estimate blood: tissue PCs for each form. The authors conclude that the resulting PCs are relatively similar to those used by (Yu, 1999a).

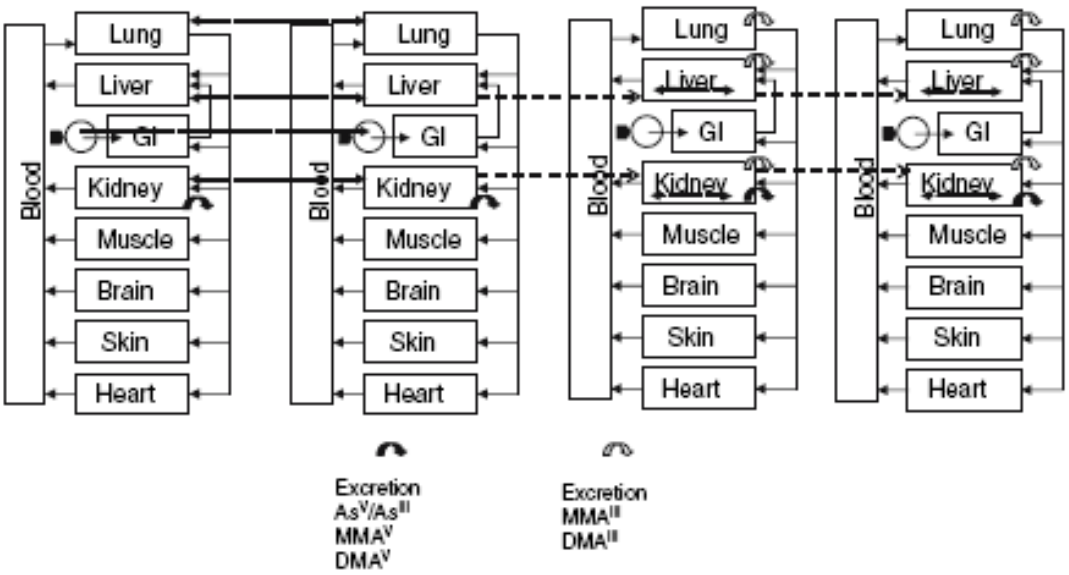


Figure E-1. Conceptual representation of physiologically based pharmacokinetic (PBPK) model.

Source: (El-Masri and Kenyon, 2008).

For the metabolites, the authors suggest inhibitory effects of As(III) on the methylation of MMA(III) to DMA(V) and of MMA(III) on the methylation of As(III) to MMA(V), which were modeled as noncompetitive inhibition (see Figure E-2).

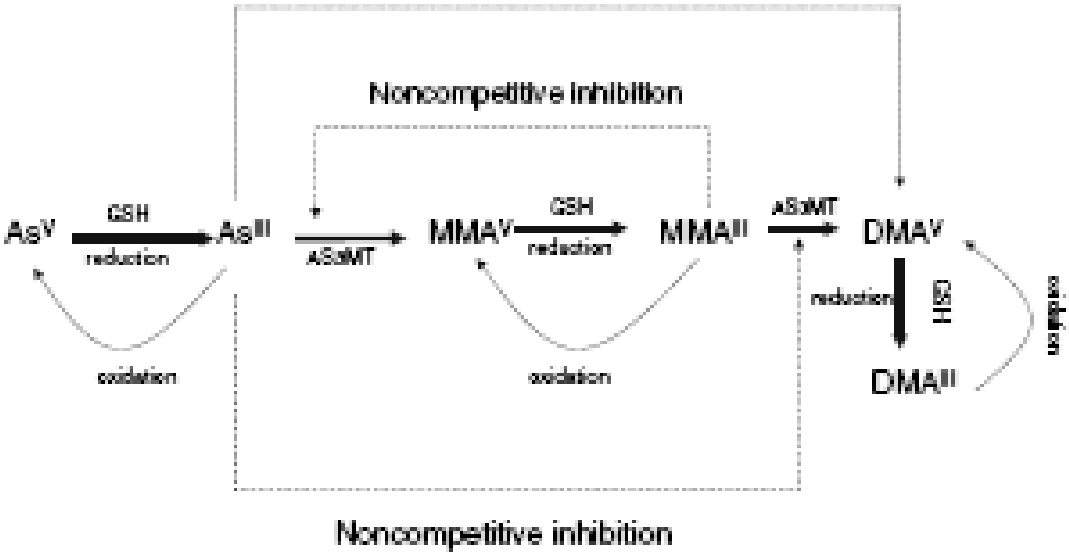


Figure E-2. Metabolism pathways describe in the literature.

Source: (El-Masri and Kenyon, 2008).

A Simulink platform was used for the simulation; MATLAB® was used for the optimization. The PBPK model was evaluated against available data in the literature (Buchet et al., 1981a); (Buchet et al., 1981b); (Lee, 1999). Overall, the evaluation of the model showed a better prediction at a low dose than at a high dose. The advantages of using the (El-Masri and Kenyon, 2008) model for risk assessment are as follows:

- This model was peer reviewed.
- This model is the most recent one published, implying it may have incorporated the most recent literature values available.
- Most of the metabolic constants used in this model were determined experimentally or were optimized from urinary excretion data following exposures to inorganic As, MMA only, and DMA only.
- Model performance was assessed using predictors analysis such as mean absolute performance error (MAPE%) and root-media-square performance error (RMSPE%).

Minor concerns for the (El-Masri and Kenyon, 2008) model for risk assessment are as follows:

- A systematic sensitivity and uncertainty analysis is needed for each parameter.
- The independent variable for time should be converted from minutes to days or weeks to facilitate lifetime exposure analysis.
- Currently, the tissue compartment volumes are constants. To facilitate modeling of lifetime exposures, the tissue volumes should be re-expressed as a body-weight fraction. This conversion is essential because tissue volume and blood flows change with age.

A possibly significant issue of the model is that MMA(III) and DMA(III) are not described outside of the lung, liver, and kidney, which is a deficiency if concentrations for these metabolites in other tissues are important in the risk assessment.

E.1.2. Analysis of (El-Masri and Kenyon, 2008)

Considering the advantages and disadvantages of the (El-Masri and Kenyon, 2008) model as described above, this section further investigates details of this model and provides additional discussion of its appropriate application. Specifically, a table of the parameter values is provided with a discussion of the appropriateness of the selection of values compared with other available parameter values in the literature. We also describe additional data sets that have become available since the publication of the paper in 2008 that could be used to modify some of the (El-Masri and Kenyon, 2008) parameters (specifically, the Michaelis constant [Km] values).

Parameter Values

Table E-2 lists the constants used in the manuscript. (Note: the table does not include any calculated parameters.) This section discusses some of the key parameters and places the values used in this model into context with other available values in the literature.

Comparison of (El-Masri and Kenyon, 2008) Parameter Values to Other Models

Table E-3 compares the binding affinity constants (Km values) and the maximum velocities (V_{max} values) used by (El-Masri and Kenyon, 2008) with those used in other models from (Yu, 1999a). (El-Masri and Kenyon, 2008) used Km values published in the literature (Zakharyan et al., 1999). (Zakharyan et al., 1999) used partially purified Chang human hepatocytes grown in culture and purified hepatocytes from rabbit to determine the Km and the V_{max} of the methyltransferase. They also assumed that the activity of arsenite methyltransferase and the MMA methyltransferase appears in the same protein but in different active centers (Zakharyan et al., 1999). Because the rabbit and Chang human hepatocyte Km's were comparable, El-Masri and Kenyon decided to use the value of the Chang human hepatocytes as the Km (3×10^{-6} M) value for their PBPK model and apply it to the equations for the conversion of MMA(III) $\rightarrow$ DMA(V); As(III) $\rightarrow$ MMA(V); and As(III) $\rightarrow$ DMA(V). (Zakharyan et al., 1999) determined an in vitro V_{max} corresponding to the maximum velocity. The same authors also showed that MMA(III) is a noncompetitive inhibitor of arsenite methyltransferase and that inorganic arsenite is a noncompetitive inhibitor of MMA(III) methyltransferase. A noncompetitive inhibitor binds to a site on the enzyme that is not the active site. The enzyme undergoes a conformational change so that product formation is inhibited. In each case, the inhibitor does not act by binding to the same active site as the respective substrates. Nevertheless, it was not possible to use the V_{max} determined by (Zakharyan et al., 1999) because, to perform the extrapolation from in vitro to in vivo, one needs to know how much enzyme protein is in the PBPK model compartment (i.e., organ) and that information is not currently available. Therefore, (El-Masri and Kenyon, 2008) used a human data set from (Buchet et al., 1981a) to estimate the V_{max}.

Table E-2. Constant list that appeared in the model code file used in the manuscript

Name	Value	Units	Descriptions of parameters
KA_AS3	0.004	min^{-1}	Oral absorption constant of As(III)
KA_AS5	0.003	min^{-1}	Oral absorption constant of As(V)
KA_DMA	0.007	min^{-1}	Oral absorption constant of DMA(V)
KA_MMA	0.007	min^{-1}	Oral absorption constant of MMA(V)
KAS3_DMA_K	3.00×10^{-6}	mol/L	Km of As(III) for its metabolism to DMA in kidney
KAS3_DMA_LI	3.00×10^{-6}	mol/L	Km of As(III) for its metabolism to DMA in liver
KAS3_MMA_K	3.00×10^{-6}	mol/L	Km of As(III) for its metabolism to MMA in kidney
KAS3_MMA_LI	3.00×10^{-6}	mol/L	Km of As(III) for its metabolism to MMA in liver
KI_AS3	4.00×10^{-5}	mol/L	Noncompetitive inhibition constant Ki of As(III)
KI_MMA	4.00×10^{-5}	mol/L	Noncompetitive inhibition constant Ki of MMA(III)
KMMA_DMA	3.00×10^{-6}	mol/L	Km of MMA(III) for its metabolism to DMA
KOX_AS	0.25	unitless	Oxidation of As(III)
KOX_DMA	0.65	unitless	Oxidation of DMA(III)
KOX_MMA	0.63	unitless	Oxidation of MMA(III)
KRED_AS	0.0025	min^{-1}	Reduction of As(V)
KRED_DMA	0.004	min^{-1}	Reduction of DMA(V)
KRED_MMA	0.0075	min^{-1}	Reduction of MMA(V)
KUR_AS	0.07	min^{-1}	Urine excretion constant of As(III) and As(V)
KUR_DMA	0.13	min^{-1}	Urine excretion constant of DMA (both forms)
KUR_MMA	0.2788	min^{-1}	Urine excretion constant of MMA (both forms)
PB_AS3	2.35	unitless	Partition coefficient brain/blood for As(III)
PB_AS5	2.4	unitless	Partition coefficient brain/blood for As(V)
PB_DMA	3.3	unitless	Partition coefficient brain/blood for DMA (both forms)
PB_MMA	2.2	unitless	Partition coefficient brain/blood for MMA (both forms)
PG_AS3	8.3	unitless	Partition coefficient GI tract/blood for As(III)
PG_AS5	2.7	unitless	Partition coefficient GI tract/blood for As(V)
PG_DMA	2.1	unitless	Partition coefficient GI tract/blood for DMA (both forms)
PG_MMA	2.2	unitless	Partition coefficient GI tract/blood for MMA (both forms)
PH_AS3	7.4	unitless	Partition coefficient heart/blood for As(III)
PH_AS5	7.9	unitless	Partition coefficient heart/blood for As(V)
PH_DMA	2.4	unitless	Partition coefficient heart/blood for DMA (both forms)
PH_MMA	2.61	unitless	Partition coefficient heart/blood for MMA (both forms)
PK_AS3	11.7	unitless	Partition coefficient kidney/blood for As(III)

Name	Value	Units	Descriptions of parameters
PK_AS5	8.3	unitless	Partition coefficient kidney/blood for As(V)
PK_DMA	3.8	unitless	Partition coefficient kidney/blood for DMA (both forms)
K_MMA	4.4	unitless	Partition coefficient kidney/blood for MMA (both forms)
PLI_AS3	16.5	unitless	Partition coefficient liver/blood for As(III)
PLI_AS5	15.8	unitless	Partition coefficient liver/blood for As(V)
PLI_DMA	3.3	unitless	Partition coefficient liver/blood for DMA (both forms)
PLI_MMA	3.3	unitless	Partition coefficient lungs/blood for MMA (both forms)
PLU_AS3	6.7	unitless	Partition coefficient lungs/blood for As(III)
PLU_AS5	2.1	unitless	Partition coefficient lungs/blood for As(V)
PLU_DMA	1.3	unitless	Partition coefficient lungs/blood for DMA (both forms)
PLU_MMA	1.3	unitless	Partition coefficient lungs/blood for MMA (both forms)
PM_AS3	7.4	unitless	Partition coefficient muscles/blood for As(III)
PM_AS5	7.9	unitless	Partition coefficient muscles/blood for As(V)
PM_DMA	2.4	unitless	Partition coefficient muscles/blood for DMA (both forms)
PM_MMA	2.61	unitless	Partition coefficient muscles/blood for MMA (both forms)
PS_AS3	7.4	unitless	Partition coefficient skin/blood for As(III)
PS_AS5	7.9	unitless	Partition coefficient skin/blood for As(V)
PS_DMA	2.4	unitless	Partition coefficient skin/blood for DMA (both forms)
PS_MMA	2.61	unitless	Partition coefficient skin/blood for MMA (both forms)
QC	5.2	L/min	Cardiac output
QB	0.63	L/min	Brain blood flow
QH	0.2	L/min	Heart tissue blood flow
QHE	0.31	L/min	Hepatic artery blood flow (~25% of total liver flow)
QK	1	L/min	Kidney blood flow
QLI	1.31	L/min	Total liver blood flow (QHE + QPV)
QM	1.8	L/min	Muscle blood flow
QPV	1	L/min	Portal vein blood flow (~75% of total liver flow)
QS	0.26	L/min	Skin blood flow
VAS3_DMA_K	2.00×10^{-6}	mol/min	V_{max} for methylation of As(III) to DMA(V) in kidney
VAS3_DMA_LI	2.00×10^{-6}	mol/min	V_{max} for methylation of As(III) to DMA in liver
VAS3_MMA_K	5.30×10^{-7}	mol/min	V_{max} for methylation of As(III) to MMA in kidney
VAS3_MMA_LI	5.30×10^{-7}	mol/min	V_{max} for methylation of As(III) to MMA in liver
VMMA_DMA	6.60×10^{-7}	mol/min	V_{max} for methylation of MMA(III) to DMA(V)
VB	1.4	L	Brain volume
VG	1.2	L	GI tract volume

Name	Value	Units	Descriptions of parameters
VH	0.35	L	Heart volume
VK	0.28	L	Kidney volume
VLI	1.82	L	Liver volume
VLU	0.56	L	Lung volume
VM	55.5	L	Muscle volume
VS	2.6	L	Skin volume

Table E-3. Comparison of Km and maximum velocity (Vmax)

Parameter	El-Masri and Kenyon (2008)	Yu (1999b)
Methylation of MMA		
V_{max} (MMA[III] ® DMA)	6.6×10^{-7} mol/min	2.67×10^{-7} mol/min
Km (MMA[III] ® DMA)	3×10^{-6} M	1×10^{-4} M
Kinh (noncompetitive inhibition)	4×10^{-5} M	NA
Methylation of As		
V_{max} (As[III] ® MMA)	5.3×10^{-7} mol/min	1.875×10^{-7} mol/min
Km (As[III] ® MMA)	3×10^{-6} M	1×10^{-4} M
V_{max} (As[III] ® DMA)	2×10^{-6} mol/min	3.708×10^{-7} mol/min
Km (As[III] ® DMA)	3×10^{-6} M	1×10^{-4} M
Kinh (noncompetitive inhibition)	4×10^{-5} M	NA

NA = not applicable.

The parameters found in the (Yu, 1999a) PBPK model either came from the literature or were fitted to the data. Briefly, the partition coefficient came from (Saady et al., 1989), the tissue volume and the blood flow came from (Reitz et al., 1990), the methylation and dimethylation were fit to the data of (Buchet et al., 1981a), and the glutathione value came from (Pilon et al., 1988). (Yu, 1999a) gave an incomplete explanation of how each parameter was determined, decreasing confidence in the parameters' accuracy. In (El-Masri and Kenyon, 2008), each parameter was rationally explained, and the limitations were well documented.

(El-Masri and Kenyon, 2008) noted that adding complex inhibitory pathways to the metabolism of arsenic and its metabolites does not yield significant differences quantitatively in model simulations at relatively low levels of arsenic exposure. The impact of the complex metabolic pathways may become evident in situations in which MMA levels are higher than those produced from iAs metabolism (El-Masri and Kenyon, 2008). In general, a PBPK model—which is a simplified representation of a biological observation—can ignore nonlimiting steps (and skip the descriptions of such steps) without altering the overall pharmacokinetics prediction. Such simplification is

useful when literature data are lacking for a specific enzymatic kinetic description. Hence, complex inhibitory pathways do not need to be included to apply the model in the low-dose regions.

Table E-4 compares the partition coefficients used in (El-Masri and Kenyon, 2008) and those used in (Yu, 1999a). The partition coefficient is an important parameter driving the distribution of parent or metabolite compounds in different compartments. As mentioned in (El-Masri and Kenyon, 2008), partition coefficients from animals such as mice are typically comparable to human values. In the (El-Masri and Kenyon, 2008) PBPK model, the partition coefficients for each tissue were estimated as described above.

Table E-4. Comparison of partition coefficients

Partition coefficients				
Compartment	**As(V)**	**As(III)**	**MMA**	**DMA**
(El-Masri and Kenyon, 2008)				
GI (small intestine)	2.7	8.3	2.2	2.1
Skin	7.9	7.4	2.61	2.4
Brain	2.4	2.4	2.2	3.3
Muscle	7.9	7.4	2.61	2.4
Kidney	8.3	11.7	4.4	3.8
Liver	15.8	16.5	3.3	3.3
Lung	2.1	6.7	1.3	1.3
Heart	7.9	7.4	2.61	2.4
(Yu, 1999a)				
GI (small intestine)	2.8	2.8	1.2	1.4
Skin	2.5	2.5	1.25	1.25
Brain	NA	NA	NA	NA
Muscle	2.6	2.6	1.8	2.8
Kidney	4.15	4.15	1.8	2.075
Liver	5.3	5.3	2.35	2.65
Lung	4.15	4.15	1.8	2.075
Heart	NA	NA	NA	NA
Fat	0.3	0.3	0.3	0.3

GI = gastrointestinal; NA = not applicable.

Note: In (Yu, 1999a), the tissue and blood partition coefficients were based on a postmortem analysis of a child weighing 16.3 kg who was poisoned [reported by (Saady et al., 1989). However, (Saady et al., 1989) only measured total As in each tissue, rather than the amount of inorganic versus methylated arsenic. Other PBPK models in the literature used optimization techniques to determine the partition coefficients (Liao et al., 2008); (Mann et al., 1996a).

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