University of Nevada, Reno

Insights on arsenic-mediated oxidative stress, mitochondrial dysfunction, and cardiovascular disease progression

A dissertation submitted in partial fulfillment of the requirements for the degree

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by

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i. Abstract

This dissertation contributes to the literature on arsenic and health, identifying four areas for specific investigation. Chapter 1 addresses arsenic in the environment examining its impact on public health in high and low exposure populations. Chapter 2 examines possibilities for ameliorating the toxicity of arsenic in real world contexts. This chapter is published in the Journal *Toxics* (doi:10.3390/toxics5040038). Chapter 3 investigates the association between metabolic syndrome and arsenic methylation capacity in a low exposure population, showing a positive association between methylation capacity and metabolic syndrome in a sample drawn from the U.S. National Health and Nutrition Examination Survey (NHANES) population. This chapter is under preparation for journal submission. Chapter 4 experimentally assesses the differential toxicity of trivalent inorganic arsenic (iAs(III)) and the metabolite trivalent monomethylarsonous acid (MMA(III)) in vascular smooth muscle cells, and our data show increased toxicity of MMA(III) relative to iAs(III) in VSMCs with mitochondrial dysfunction as a central component of toxicity. This chapter is published in the journal *Toxicology in Vitro*

(https://doi.org/10.1016/j.tiv.2016.06.006). Chapter 5 provides a summary, conclusions, and recommendations for future research.

Methodologically, this dissertation is composed of a review and synthesis of published data, a secondary data analysis from an epidemiology perspective, and an original study of experimental data. Each chapter addresses gaps in the current literature pertaining to various aspects of arsenic and cardiovascular disease. The dissertation as a whole contributes to our understanding of a significant and ubiquitous environmental toxin.

This research is important and innovative because it applies diverse methodologies to study arsenic and cardiovascular disease. The selected methodologies are relevant in the fields of environmental science and epidemiology. This research has implications for millions of individuals exposed to arsenic through contaminated ground water around the world as well as among select arsenic-exposed populations in the U.S., and provides the foundation for future exploration of the selective toxicity of arsenic and its metabolites to mitochondria, the relevance of arsenic methylation capacity as a risk factor for the health effects of arsenic, and a framework for developing

antioxidant solutions to the global problem of arsenic induced cardiovascular toxicity.

In chapter 2 we reviewed 25 studies that assessed the effects of non-enzymatic antioxidants against arsenic exposed mitochondria and/or cardiovascular cells and tissues. We report that arsenic impairs all aspects of mitochondrial function and mitigates apoptosis by elevating reactive oxygen species (ROS). The antioxidants presented in this review largely prevented arsenic-induced pathology *in vivo* and *in vitro*. Compared to arsenic treatment alone, co-treatment with phytonutrient antioxidants restored cardiac function, reduced ROS levels, restored antioxidant activities, reduced apoptosis, reduced calcium overload, restored ATP content, and restored the activity of mitochondrial complexes. We suggest that future studies directly compare antioxidant compounds against arsenic-induced cardiovascular dysfunction, and we encourage the development of mitochonrially targeted antioxidant nanoformulations to improve antioxidant bioavailability.

In chapter 3 we report a positive association between increased arsenic methylation and metabolic syndrome in women with normal BMI, and among obese women after controlling for statistically significant and literature justified

covariates. Our data suggest that gender and BMI modify the association between arsenic methylation and metabolic syndrome. Additionally, we report that arsenic methylation patterns in our sample are similar to reports of methylation capacity in populations with significantly higher arsenic exposure. Based on these results, we suggest that future studies evaluate the effectiveness of the current maximum contaminant level (MCL) for arsenic on preclinical biomarkers for cardiovascular disease in a model that establishes causality.

In chapter 4 we demonstrate increased toxicity of the arsenic metabolite MMA(III) relative to iAs(III) in VSMCs. We report for the first time that MMA(III) promotes mitochondrial dysfunction and oxidative stress in VSMCs, and that these deficits stem from the generation of mitochondrial and non-mitochondrial ROS following exposure to MMA(III). This work warrants future studies that probe for molecular mechanisms by which MMA(III) selectively targets mitochondria and elicits the release of superoxide in VSMCs.

The results from chapter 4 (increased toxicity of methylated arsenic species) support our results from chapter 3, in which we demonstrate that increased arsenic methylation is associated with increased odds of metabolic syndrome, presumably due to the elevated presence of toxic methylated species

that result from increased arsenic methylation. Data from chapters 3 and 4 support the premise that increased arsenic methylation increases the concentrations of toxic methylated arsenic species in the body, resulting in increased rates of ROS production, and contributing to disease status. Our results from chapter 2 (antioxidant amelioration of arsenic-induced cardiovascular dysfunction) provide recommendations for future research on the prevention of arsenic-induced cardiovascular disease.

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Chapter 1. Arsenic: geographical distribution and health implications

i. Arsenic in the environment

Arsenic is a metalloid with possible oxidation states -3, 0, +3 and +5, primarily found as inorganic arsenite (iAs(III)) in reducing conditions and inorganic arsenate (iAs(V)) under oxygenated conditions. Inorganic arsenic compounds can be trivalent (arsenic trioxide, arsenic trichloride, sodium arsenite, and arsenic disulfide) or pentavalent (arsenate and arsenic pentoxide) (**Figure 1**). In nature, arsenic primarily occurs in sulfide form complexed with one of over 200 mineral species [1].

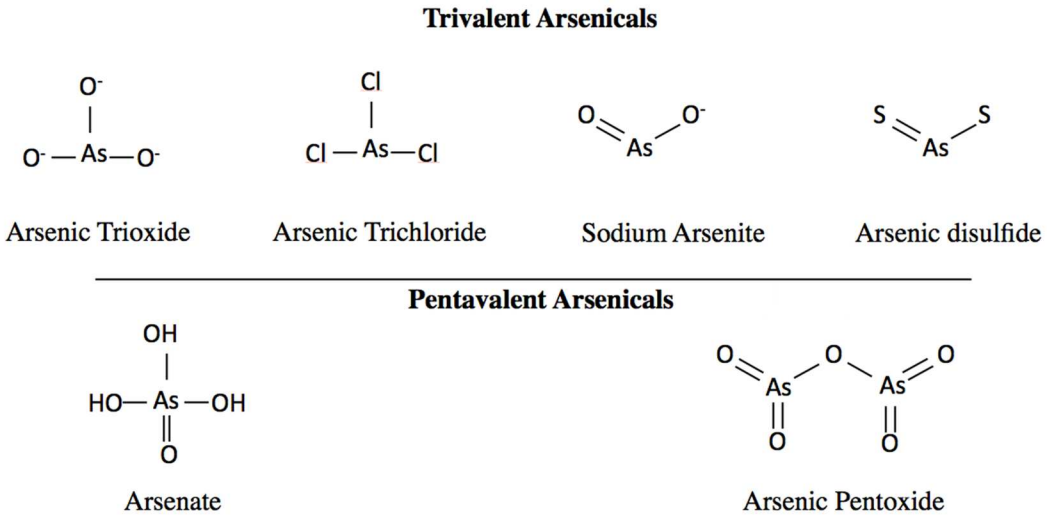


Figure 1. Trivalent and pentavalent arsenic species

i.i. Arsenic in Groundwater

The release of arsenic to groundwater varies as a function of the underlying geology and geochemistry of the aquiferous basin, and climate of the

surrounding area. Geologic formations that contain arsenic include sulfide minerals, iron oxides, felsic volcanic rock, and shale [2]. These formations can release arsenic to groundwater in the presence of elevated pH, oxic or sulfidic redox conditions, naturally occurring or applied phosphate, and geothermal waters [2]. Catalyzing bacteria and intense evaporative conditions can further concentrate arsenic in groundwater sources [2].

Human activities such as mining, phosphate fertilizer application, and improper disposal of arsenical waste can increase regional arsenic concentrations. Perhaps the largest anthropogenic impact on arsenic availability occurs through the mismanagement of water resources. Global water demands continue to rise in proportion to growing populations, resulting in decreased surface water supplies and a growing dependence on groundwater [3]. As shallow aquifers are tapped beyond replacement levels, arsenic concentrates in groundwater basins. Attempts to tap deeper aquifers can result in drawdown in which arsenic infiltrates previously uncontaminated groundwater [4].

i.ii. Global distribution

There are many past and present instances of arsenic exposure throughout the world. Inorganic arsenic is commonly found in tube wells in Bangladesh

within the range of 200 to 300 ppb, and has been documented in excess of 1,000 ppb, continuing to expose about 50 million residents [5]. Water in Antofagasta, Chile containing 470 to 770 ppb of arsenic was supplied from the Andes Mountains via the Tocance River from 1959 to 1970, exposing 130,000 inhabitants until a water treatment plant was installed [1]. The Cordoba Provence in northern Argentina has reported water ranging from 100-3,810 ppb arsenic resulting from volcanic conditions and thermal springs [6]. Other notable instances of arsenic exposure have occurred in Taiwan, Japan, West Bengal, Mexico, and various regions of the Western United States [7].

i.iii. United States

Pockets of arsenic-endemic groundwater are present throughout the United States (U.S.). Although inorganic arsenic levels are regulated in municipal waters by the U.S. Environmental Protection Agency (U.S. EPA) at 10 parts per billion (ppb), exposure to higher concentrations of arsenic still occur among U.S. populations reliant on well water. For example, a survey of 102 homes using domestic wells in rural areas of Nevada found an average of 356 ppb arsenic pre-treatment and 87 ppb arsenic following reverse osmosis treatment [8], and arsenic concentrations were measured between 10 and 61 ppb on rural

reservation lands in Arizona [9]. Additionally, researchers found that a population of 250 was drinking well water containing between 180 and 210 ppb inorganic arsenic in Millard County, Utah [10], and residents of Lane County, Oregon consumed well water that ranged from 5 to 1,700 ppb arsenic due to underlying sedimentary and volcanic rock [11].

ii. Health effects of arsenic

ii.i. Arsenic bioavailability and metabolism

Bioavailability refers to the percentage of the external dose that is absorbed and reaches systemic circulation. Studies conducted on human volunteer subjects reveal that between 80% and 90% of a single dose of ingested arsenite or arsenate is absorbed by the human GI tract [12-14]. However, arsenic absorption varies at the individual level and is affected by the presence of food, stomach acidity, and GI tract flora [15-17].

Ingested arsenic enters circulation, travels to organs and tissues, and accumulates in the kidneys, liver, hair, skin, and nails [18,19]. Inorganic arsenic is reduced from arsenic (V) to arsenic (III) in blood plasma and arsenic methyltransferase enzymes in the liver mediate the methylation process with S-adenosylmethionine as a methyl donor [18]. In these reactions, methyl groups are

sequentially added to inorganic arsenic species forming the organic arsenic species monomethyarsonous acid (MMA) and dimethylarsonous acid (DMA) (**Figure 2**).

Arsenic methylation capacity can be measured as the proportion of methylated arsenicals in urine or as a ratio of MMA:iAs (termed primary methylation index or PMI) and ratio of DMA:MMA (termed secondary methylation index or SMI). Average relative distribution of urine metabolites is 10-30% inorganic arsenic, 10-20% MMA, and 60-80% DMA [20]. However, urine arsenic distribution varies somewhat as a function of genetic polymorphisms controlling methyltransferases [21].

Arsenic methylation facilitates urinary excretion, yet it also increases the bioavailability of toxic methylated arsenicals [22-27]. Indeed, arsenic (III) species are more toxic than arsenic (V) species, and the metabolites MMA(III) and DMA(III) have been shown to be 26 and 100 times more toxic to certain cell types compared to iAs(III), respectively [26]. The elevated toxicity of trivalent and methylated arsenicals is primarily a function of their increased reactivity with biological molecules.

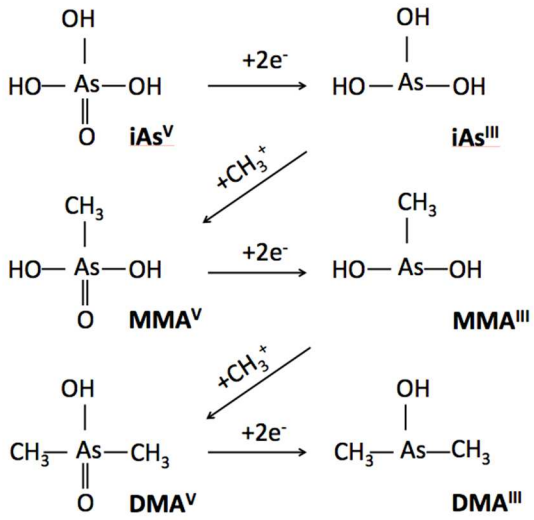


Figure 2. Arsenic biomethylation

ii.ii. Toxicity

Both acute and chronic arsenic toxicity manifest in virtually all body systems. The acute lethal dose of arsenic in humans is approximately 0.6 mg/kg/day [28]. Acute arsenic exposure results in vomiting, abdominal colic, and diarrhea [16]. In rare cases, arsenic poisoning can cause death through massive fluid loss due to secretions from the gastrointestinal tract, which in turn cause severe dehydration, reduced blood volume, and circulatory collapse [28]. Prolonged exposure to non-lethal doses of arsenic can cause thickening of the skin of the palms (keratosis), skin pigmentation (melanosis), and systemic disease of the liver, kidney, nervous system, and cardiovascular systems, as well as squamous–cell carcinoma [16].

Arsenic species are known to inhibit more than 200 enzymes [29]. Arsenic (III) interferes with enzyme systems by binding to –SH and –OH groups [30], and an affinity for sulfur allows arsenic to accumulate in keratin tissue [18]. Arsenic species can compete with phosphate, uncoupling oxidative phosphorylation and disrupting ATP production [31]. Arsenic may also replace phosphorus in DNA, inhibit DNA repair mechanisms [32] and interfere with selenium in human metabolism [33].

ii.iii. Mortality and cancer

Chronic exposure to arsenic can result in increased overall mortality and risk of cancer, diabetes, organ toxicity and cardiovascular disease. A prospective cohort study in Bangladesh assessed all cause and chronic disease mortality among over 11,000 subjects at the individual level. Exposures in this population were often in the range of 200 to 300 ppb. Argos et al. (2010) reported that 21.4% of all deaths and 23.5% of deaths associated with chronic disease could be linked to arsenic exposure in drinking water [34]. Dose response mortality patterns have been seen at the ecological level for cancers of the lung, skin, bladder, and kidney in populations exposed to arsenic in excess of 50 ppb [35-38].

In addition to cancer mortality, the incidence of lung, bladder, kidney and liver cancers, as well as basal cell and squamous cell carcinomas have been linked to arsenic exposure in highly exposed populations of Taiwan, Japan, West Bengal, Bangladesh, Chile, and Argentina [1,39-41].

ii.iv. Diabetes and organ toxicity

Elevated diabetes risk has been demonstrated among arsenic-exposed populations of Bangladesh [42,43], and diabetes has been observed at a two-fold increase in arsenic-exposed areas compared to the rest of Taiwan [44]. Arsenic accumulates in the liver and can contribute to cirrhosis, portal hypertension, fatty degeneration, and jaundice [45-47]. The kidneys, the major site of arsenic excretion, accumulate arsenic where it damages capillaries, tubules, and glomeruli, and increases the risk of renal failure [48].

ii.v. Cardiovascular disease

Epidemiological studies have convincingly established an association between arsenic exposure above 50 ppb and hypertension [43], atherosclerosis [49], and ischemic heart disease [50]. Blackfoot disease, a peripheral vascular disease involving progressive loss of circulation to the extremities and often

resulting in amputation, has been linked to arsenic exposure in regions of Taiwan [51]. Additionally, carotid atherosclerosis increases dose dependently with arsenic exposure [49]. Furthermore, high pulse pressure, an indicator of arterial stiffness and risk factor for atherosclerosis, is significantly higher among arsenic exposed compared to unexposed subjects in Bangladesh [52].

The cardiovascular effects of arsenic are not limited to highly exposed populations. Indeed, a prospective cohort study in the U.S. compared the hazard ratios of cardiovascular disease (CVD) and coronary heart disease incidence among low (5.7 ppb) to moderately (15.7 ppb) arsenic exposed subjects [9]. The authors report hazard ratios of 1.32 (95% CI: 1.09 -1.59) for incident CVD and 1.30 (95% CI: 1.04 -1.62) for incident coronary heart disease [9]. Statistically significant hazard ratios were also reported for CVD, coronary heart disease, and stroke mortality among moderate compared to low exposure subjects [9].

ii.vi. Metabolic syndrome

Metabolic syndrome is a risk factor for cardiovascular disease and diabetes. Metabolic syndrome is defined as the presence of at least 3 out of 5 components including elevated fasting glucose, hypertension, elevated triglycerides, large waist circumference, and low HDL cholesterol. Wang et al.

(2007) demonstrated that inorganic arsenic exposure was associated with a clinical diagnosis of metabolic syndrome, elevated plasma glucose, and elevated blood lipids among residents of an industrial region of Taiwan exposed to arsenic both occupationally and through water sources measured up to 16 ppb [49]. Additionally, Chen et al. (2012) reported that a high proportion of urine DMA was associated with increased risk of metabolic syndrome among a population in Taiwan exposed to median arsenic concentrations of 700-930 ppb [53]. Elevated blood pressure was positively associated with elevated urine concentrations of DMA in the U.S. NHANES 2009-2012 population [54], and large waist circumference was associated with a high proportion of urine DMA among participants of the Strong Heart Study, a rural cohort from the southwest U.S. [55].

ii.vii. The role of mitochondria in cardiovascular disease and metabolic disorder

The cardiovascular toxicity of arsenic is mediated, in part, through mitochondrial dysfunction. Due to high-energy demand, cardiovascular tissues are heavily enriched with mitochondria and are thus particularly susceptible to mitochondrial toxins [56]. The respiratory chain is a critical mitochondrial mechanism by which toxic agents act on mitochondria, leading to decreased ATP production, reduced mitochondrial

transmembrane potential, and increased reactive oxygen species (ROS) [56]. Arsenic has exhibited toxic effects in the mitochondria of vascular smooth muscle [57], myocardial cells [58], and vascular endothelial cells [59]. Arsenic is associated with mitochondrial dysfunction through mechanisms such as elevated ROS, induction of apoptosis, and Ca^{2+} overload [60].

ii.viii. Arsenic as cancer treatment and the role of antioxidants

Arsenic is a recognized carcinogen. Arsenic-induced ROS cause genetic mutations and cancer through DNA damage, activation of oncogenic kinases, and activation of lipids and proteins that inactivate DNA repair mechanisms [61,62]. Paradoxically, arsenic trioxide has been used as a therapeutic agent in the treatment of acute promyelotic leukemia (APL). APL is a subtype of acute myeloid leukemia (AML) that is genetically characterized by a specific chromosomal translocation that yields the promyelotic leukemia/retinoic acid receptor alpha (PML/RARA) fusion gene - a DNA-binding transcription factor. Arsenic targets the PML moiety of PML/RARA and disrupts PML unclear bodies, which regulate stem cell self-renewal [63]. Specifically, arsenic was found to bind to protein kinase M2 (PKM2), located on the surface of PML/RARA. PKM2 promotes aerobic glycolysis ("Warburg effect") leading to tumorogenisis and cancer cell proliferation. Arsenic reduces PKM2 activity, thus

inhibiting cancer cell growth [64].

Despite the benefits, the clinical use of arsenic trioxide is severely limited due to arsenic's severe cardiovascular side effects. A growing body of evidence suggests that antioxidant compounds may ameliorate arsenic's toxicity to the cardiovascular system. Antioxidant phytonutrients are naturally occurring chemicals in plants such as vegetables, grains, legumes, seeds, fruits, leaves, flowers, and bark. Polyphenols are a major class of phytochemicals that have non-enzymatic antioxidant activities and scavenge free radicals and oxidants such as superoxide ($0_2 \cdot$), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2) [65]. As ROS are a major contributor to arsenic-induced toxicity, it is possible that polyphenols and other antioxidants could be employed against the deleterious effects of arsenic in the cardiovascular system.

iii. Addressing gaps in the literature

Despite a growing body of evidence on the hazards of arsenic exposure, there are several important gaps in the literature. No systematic review has explored the protective actions of food-based antioxidants against arsenic-induced mitochondrial toxicity. Therefore, chapter 2 reviews the available literature on *in vivo* and *in vitro* studies using antioxidants to counteract arsenic-induced cardiovascular toxicity. We report on 22 antioxidant compounds with free radical scavenging or chelating activities

employed against arsenic-mediated toxicity in cardiovascular cells and tissues, and suggest future directions for research aimed at ameliorating the harmful effects of arsenic in a real-world context.

In light of arsenic-induced cardiovascular dysfunction and evidence that methylation capacity contributes to arsenic's toxicity, chapter 3 investigates the association between arsenic methylation capacity and metabolic syndrome. While arsenic methylation capacity is known to be associated with a diagnosis of metabolic syndrome in high exposure populations, it is unknown if arsenic methylation contributes to metabolic syndrome in a low exposure population. To answer this question we conducted a secondary data analysis using data from the U.S. National Health and Nutrition Examination Survey (NHANES). Chapter 3 reports a positive association between arsenic methylation capacity and metabolic syndrome in a population with low arsenic exposure represented by the U.S. NHANES population. Methylation capacity contributes to the extent to which toxic arsenic metabolites enter and remain in systemic circulation. Arsenic metabolites are hypothesized to be major contributors to disease, however, many of the mechanisms pertaining to the toxicity of different arsenic species remains to be elucidated. Chapter 4 experimentally assessed the differential toxicity of iAs(III) and the metabolite MMA(III) in VSMCs. This cell type was selected because it is highly enriched with mitochondria and involved in CVD

dysfunction. Therefore, in chapter 4, we report increased toxicity of MMA(III) relative to iAs(III) in VSMCs with mitochondrial dysfunction as a central component of toxicity. This outcome adds to the growing body of literature on mechanistic differences between arsenic species.

iv. Methodology

This dissertation uses three different methodological approaches. Chapter 2 is a review and synthesis of published data. We conducted a literature search of MEDLINE, web of science, and Google scholar for the terms and relevant variations of "mitochondria" "toxicity" "cardiovascular" "arsenic" and "antioxidant." We excluded reviews and publications not available in English. This chapter tested the hypothesis that food-based antioxidants are highly effective against arsenic induced mitochondrial toxicity in cardiovascular cells and tissues. The selected 25 studies assessed the effects of non-enzymatic antioxidants against arsenic exposed mitochondria and/or cardiovascular cells and tissues.

Chapter 3 is a secondary data analysis using U.S. NHANES data to draw epidemiological conclusions. NHANES uses a complex, probability sampling design to select a nationally representative sample of subjects to

participate in each 2-year cycle. NHANES personnel collected demographic information via survey, and a physical examination was performed on all subjects. Biological samples were collected by NHANES personnel from nationally representative subsamples. In our study, we included a total of 957 subjects over the age of 20 who had complete data on all relevant variables.

We used logistic regression to assess the relationships between measures of methylation capacity with metabolic syndrome. Covariates were evaluated in the literature and statistically using Mann-Whitney-U test, Kruskal Wallace test, χ^2 test, or student-t test, and statistically relevant or biologically justified covariates were included in the final adjusted model. We report a positive association between arsenic methylation and metabolic syndrome in women with normal BMI and obese women, and that both gender and BMI modify the effect of arsenic methylation on metabolic syndrome.

Chapter 4 is an experimental laboratory study of the differential toxicity of iAs(III) and MMA(III) on VSMCs. We used a variety of methodological approaches to assess cell viability, cell death, mitochondrial respiration, mitochondrial ATP levels, reactive oxygen species (ROS), and mitochondrial morphology and content. We report increased toxicity of MMA(III) relative to iAs(III) in VSMCs.

In summary, this dissertation is composed of a review and synthesis of published data, a secondary data analysis from an epidemiology perspective, and original experimental data, all of which address gaps in the literature pertaining to various aspects of arsenic and cardiovascular disease. We conclude with a short chapter summarizing our findings with recommendations for future research pertaining to the selective toxicity of arsenic and its metabolites to mitochondria, the relevance of arsenic methylation capacity as a risk factor for the health effects of arsenic, and a framework for developing antioxidant solutions to the global problem of arsenic-induced cardiovascular toxicity.

Chapter 2. Antioxidants protect against arsenic induced mitochondrial cardio-toxicity

i. Abstract

Arsenic is a potent cardiovascular toxicant associated with numerous biomarkers of cardiovascular diseases in exposed human populations. Arsenic is also a carcinogen, yet arsenic trioxide is used as a therapeutic agent in the treatment of acute promyelotic leukemia (APL). The therapeutic use of arsenic is limited due to its severe cardiovascular side effects. Many of the toxic effects of arsenic are mediated by mitochondrial dysfunction and related to arsenic's effect on oxidative stress. Therefore, we investigated the effectiveness of antioxidants against arsenic induced cardiovascular dysfunction. A growing body of evidence suggests that antioxidant phytonutrients may ameliorate the toxic effects of arsenic on mitochondria by scavenging free radicals. This review identifies 22 antioxidants that can effectively reverse mitochondrial dysfunction and oxidative stress in cardiovascular cells and tissues. In addition, we propose that antioxidants have the potential to improve the cardiovascular health of millions of people chronically exposed to elevated arsenic concentrations through contaminated water supplies or used to treat certain types of leukemia. Importantly, we identify conceptual gaps in research and development of new mito-protective antioxidants and suggest avenues for future research to

improve bioavailability of antioxidants and distribution to target tissues in order reduce arsenic-induced cardiovascular toxicity in a real-world context.

ii. Introduction

ii.i. Arsenic and cardiovascular disease

Exposure to arsenic through contaminated ground water is widespread in certain regions of many countries including Bangladesh, India, and China [18]. Arsenic is a potent cardiovascular toxicant; epidemiological evidence has linked arsenic exposure to ischemic heart disease, cerebrovascular disease, atherosclerosis, and hypertension in exposed human populations [50,66,67].

Arsenic has been characterized as a strong carcinogen [36]. Arsenic-induced reactive oxygen species (ROS) cause genetic mutations and cancer by promoting DNA damage, activating oncogenic kinases, and activating lipids and proteins that inactivate DNA repair mechanisms [61,62]. Paradoxically, arsenic trioxide has been used as a therapeutic agent in the treatment of acute promyelotic leukemia (APL). APL is a subtype of acute myeloid leukemia (AML) that is genetically characterized by a specific chromosomal translocation that yields the promyelotic leukemia/retinoic acid receptor alpha (PML/RARA) fusion gene - a DNA-binding transcription factor [63]. Arsenic targets the PML moiety of

PML/RARA and disrupts PML nuclear bodies, which regulate stem cell self-renewal [63]. Specifically, arsenic was found to bind to cysteine thiols on pyruvate kinase M2 (PKM2), located on the surface of PML/RARA [64]. PKM2 is a glycolytic enzyme that promotes aerobic glycolysis (the "Warburg effect") leading to tumorigenesis and cancer cell proliferation. Arsenic reduces PKM2 activity, thus inhibiting cancer cell growth [64].

Arsenic also prevents cancer cell proliferation in human breast MCF-7 cancer cells by binding to thioredoxin reductase (TrxR) in the thioredoxin (Trx) system [68]. The Trx system, which contains NADPH, TrxR and Trx, is an important thiol-dependent electron donor system in the cell that regulates numerous cell functions including cell viability and proliferation [69,70]. As Trx and TrxR are overexpressed in many aggressive tumors, the Trx system has become an important target for cancer drug development [71,72]. Lu et al. (2007) demonstrated that arsenic trioxide irreversibly inhibits mammalian TrxR by binding at the N-terminal redox–active dithiol and the C-terminal selenothiol-active sites [68]. Despite the benefits, the clinical utility of arsenic trioxide is limited due to arsenic's severe cardiovascular side effects such as QT-prolongation, ventricular arrhythmias, Torsades de Pointes, and sudden cardiac death [73-75].

ii.ii. Mitochondria in cardiovascular disease

The cardiovascular toxicity of arsenic is mediated, in part, through mitochondrial dysfunction. Due to their significant energetic requirements, cardiovascular tissues generally have high mitochondrial densities and are thus particularly susceptible to mitochondrial toxins [56]. The electron transport system (ETS) is a critical mitochondrial mechanism. Components of the ETS are particularly susceptible to the toxic effects of arsenic, leading to decreased ATP production, reduced mitochondrial transmembrane potential, and increased ROS [56].

ii.iii. Oxidative stress

ROS cause oxidative modifications of cellular macromolecules including proteins, lipids, and polynucleotides. ROS include superoxide radical ($O_2^{\cdot}$) which is converted into hydrogen peroxide (H_2O_2) spontaneously or via dismutation catalyzed by superoxide dismutase (SOD) [76]. $O_2^{\cdot}$ also reacts with nitric oxide (NO) to generate peroxynitrite ($ONOO^-$), an oxidant and reactive nitrogen species. Electron leakage from the mitochondrial electron transport chain (ETC) to molecular oxygen generates a steady stream of O_2^-, and other enzymes

(NADPH, oxidases, lipoxygenase, cyclooxygenase, cytochrome P450s, and xanthine oxidases (XOs)) also participate in ROS generation [77,78].

Intrinsic antioxidants such as SOD, catalase, and glutathione peroxidase (GPx) are the first line of defense against oxidative stress. Excessive ROS or diminished innate antioxidant capacity result in increased oxidative stress and are associated with a multitude of downstream effects as well as disease initiation and progression [79].

ii.iv. Antioxidant phytonutrients

A growing body of evidence demonstrates the mitochondrial toxicity of arsenic in cardiovascular tissues. Importantly, antioxidant phytonutrient compounds counteract the toxic effects of arsenic on mitochondria [80]. Antioxidant phytonutrients are naturally occurring chemicals in plants such as vegetables, grains, legumes, seeds, fruits, leaves, flowers, and bark. Polyphenols are a major class of phytochemicals that have non-enzymatic antioxidant activities and scavenge free radicals such as $O_2^{\cdot}$, and H_2O_2 [65].

The present review focuses on *in vivo* and *in vitro* studies of arsenic on cardiovascular tissues and cells in which the mitochondrial endpoints of membrane potential, ATP production, ROS, respiratory chain activity, and

antioxidant content and activity were analyzed. In particular, we reviewed a comprehensive list of antioxidant phytonutrients used to counteract mitochondrial toxic (mito-toxic) effects of arsenic. The antioxidants discussed here may have a therapeutic value for both individuals chronically exposed to arsenic through groundwater sources as well as patients who require arsenic trioxide therapy for APL. Importantly, we suggest future directions for research and clinical interventions applying antioxidant therapy to revert mitochondrial dysfunction, oxidative stress and cell death of cardiovascular tissues in humans exposed to high levels of arsenic trioxide.

ii.v. Mitochondria

ii.v.i. Energy production

The primary function of mitochondria is to produce energy in the form of ATP through oxidative phosphorylation [81]. Glucose is broken down into pyruvate to generate NADH, which is oxidized in the presence of oxygen (aerobic respiration) or in limited oxygen conditions (anaerobic fermentation) [56]. Pyruvate is actively transported through the inner mitochondrial membrane into the matrix where it is oxidized and combined with coenzyme A to generate CO_2, acetyl CoA, and NADH [82]. During aerobic respiration, acetyl CoA is then

oxidized to generate CO_2, NADH and $FADH_2$, which donate electrons for the electron transport chain, and ATP. NADH and $FADH_2$ are also produced via glycolysis in the cytosol and imported to the mitochondrial matrix [56]. Electrons from NADH and $FADH_2$ are used in the process of oxidative phosphorylation to donate electrons for the respiratory chain during oxidative phosphorylation [56].

The respiratory chain consists of 5 large protein complexes called NADH-Q oxidoreductase, Q-cytochrome c oxidoreductase, cytochrome c reductase, cytochrome-c-oxidase, and ATP synthase (complexes I, II, III, IV, and V) [56]. The transfer of electrons sets up an electron gradient used to pump protons across the inner membrane space. Protons move down their concentration gradient back to the matrix, where they combine with O_2 to form H_2O and serve to power ATP synthase (Complex V) to facilitate the generation of ATP from ADP and inorganic phosphate [81].

Despite the overall efficiency of the respiratory chain, a small amount of electron leakage occurs at mitochondrial complexes I and III. This charge leakage can reduce oxygen to form reactive oxygen species such as $O_2^{\cdot}$, H_2O_2, and $^{\cdot}OH$ [56]. Deficiencies in mitochondrial energy production can be demonstrated in an experimental setting through decreased ATP content, decreased activity of

mitochondrial complexes, and elevated ROS, particularly mitochondrial superoxide, suggesting decreased respiratory chain efficiency [56].

ii.v.ii. Calcium storage

In addition to other intracellular storage deposits, calcium is transiently stored in the mitochondria and used for signal transduction and regulation of intracellular reactions. Calcium is taken up into the mitochondrial matrix by the calcium uniporter in the inner mitochondrial membrane, which is driven by mitochondrial membrane potential [83]. Calcium can later be released via a sodium-calcium exchange protein or by a change in the membrane potential [83]. Dysfunction in mitochondrial calcium status, such as Ca^{2+} overload, can result in elevated oxidative stress through Ca^{2+}-stimulated NO production, Ca^{2+}-induced cardiolipin peroxidation, Ca^{2+}- induced mitochondrial permeability pore opening with consequent release of cytochrome c and GSH-antioxidative enzymes, and activation of Ca^{2+}-calmodulin dependent protein kinases [83]. Additionally, the rise in mitochondrial ROS can alter Ca^{2+} dynamics and increase Ca^{2+} surge. Thus the reciprocal interaction between Ca^{2+} induced ROS increase and ROS-modulated Ca^{2+} upsurge can create a self-amplified loop [83].

ii.v.iii. Apoptosis

Mitochondrial-mediated apoptosis is an important mechanism of programed cell death. Pro-apoptotic proteins on the surface of mitochondria detect mitochondrial damage and activate BAX proteins, which "punch" pores in the outer mitochondrial membrane, allowing cytochrome c to be released into the cytoplasm, where it binds to apoptotic protease activating factor-1 (Apaf-1) [82,84]. The interaction of these proteins forms apoptosomes, which bind and activate caspase-9, and subsequently cleave mitochondrial membrane proteins and result in phagocytosis [82,84]. Elevated levels of BAX, Apaf-1, and caspases 3, 8, and 9 all suggest an increase in apoptosis. Other biomarkers of apoptosis include upregulation of TGF-β, a profibrogenic cytokine that indicates repressed cell proliferation and apoptosis, and phosphorylation of the NF-κB pathway.

ii.v.iv. Membrane potential

Mitochondria closely regulate membrane potential, which is critical for mitochondrial functions including the electron transport chain and calcium homeostasis [56]. A significant loss of membrane potential depletes the cell of ATP and results in cell death [85]. Dysfunctional membrane potential can be measured fluorescently by the uptake of ratiometic mitochondrial membrane

specific dye JC-1, and by measuring the membrane pore permeability and Na^+/K-ATPase activity.

Protein kinases are translocated to mitochondria in response to oxidative stress, where they regulate membrane potential, impact growth factors, and lead to phosphorylation of respiratory chain proteins. The mitogen-activated protein kinase (MAPK) cascade includes the subfamilies p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). p38 MAPK signaling regulates cell death, in part, by initiating the translocation of BAX from the cytosolic to mitochondrial compartments [85]. Activated JNK stimulates apoptosis by inhibiting the anti-apoptotic Bcl-2 and Bcl-xl, leading to cytochrome c release and subsequent induction of apoptosis. Increased ERK1/2 activity leads to ERK1/2 colocalization to the mitochondria where it promotes autophagy and mitochondrial degradation [86]. This creates a self-amplifying loop of mitochondrial dysfunction in which mitochondrially-derived ROS activates ERK1/2, which translocates to the mitochondria where it damages ATP synthase function, reduces mitochondrial membrane potential, and causes cytochrome c release, leading to further ROS release [85].

ii.v.v. Source of ROS

Mitochondria are an important source of ROS, which play a significant role in cell signaling [56]. As previously mentioned, some ROS are produced as a result of electron leak that occurs due to the incomplete flow of electrons in complex I and in complex III. ROS levels are typically low and are maintained in balance through the activity of intrinsic antioxidants, which scavenge for ROS. For example, superoxide dismutases (SODs) convert O_2^- to H_2O_2, and catalase subsequently converts H_2O_2 to water. Another endogenous antioxidant is glutathione reductase (GSH), which donates a reducing equivalent ($H^+ + e^-$) to neutralize ROS. By donating an electron, GSH becomes reactive and binds to another reactive GSH to form glutathione disulfide (GSSG). Oxidized glutathione can be converted back to its reduced state using NADPH as an electron donor, and the ratio of GSH/GSSG is often used as a measure of oxidative stress [87]. Glutathione S-transferase (GST) utilizes GSH as a substrate and participates in the metabolism of xenobiotics [88], and glutathione peroxidase (GPx) and thioredoxin reductase (TR) catalyze interconversion and equilibrium among reduced/oxidized species [78].

Excessive ROS or decreased content/activity of endogenous antioxidants result in oxidative damage to cells and phospholipid membranes [81]. Studies

have used a variety of methods to assess oxidative stress. A direct measure of ROS can be obtained by measuring the fluorescence of 2',7'-dichlorofluorescin diacetate (DCFH-DA) to 2-, 7'-dichlorofluorescein (DFA) [89]. DCFH-DA readily diffuses into cells, is hydrolyzed to H_2 DCF (which is not membrane permeable), and then is oxidized by H_2O_2 and other ROS to the fluorescent compound DFA. The fluorescence of DFA is proportional to the intracellular concentration of combined H_2O_2, $ONOO^-$, and OH^- [90].

ii.v.vi. Arsenic and cysteine thiol binding

Arsenic is considered a "sulfhydryl-reactive metalloid" [91]. The affinity of arsenic for free sulfhydryl groups results in certain mechanisms of toxicity, including binding and depletion of GSH pools, as well as binding and inactivation of sulfhydryl-rich proteins [88]. This cysteine thiol binding-mediated inactivation of specific proteins is central to the ability of arsenic to both induce metallothionein biosynthesis [92] and elevate ROS generation via inhibition of the antioxidant enzyme thioredoxin reductase [68]. The depletion of GSH titers by arsenic may also influence arsenic methylation rates, leading to variable rates of metabolic activation in certain tissues [93]. Protein cysteine thiol binding in particular tissues, including cardiac muscle, results in decreased proteinaceous

antioxidant activity, leading to accumulation of ROS and attendant pathological consequences [94-97].

ii.v.vii. ROS and the Nrf2 pathway

Arsenic's affinity for sulfhydryls also contributes to the activation of Nuclear factor erythroid 2-related factor (Nrf2) [89]. Kelch ECH (Keap-1) anchors Nrf2 in the cytoplasm in quiescent conditions. Arsenic binds to Keap-1 cysteine residues, leading to Nrf2 dissociation and translocation to the nucleus [98,99]. Nrf2 activation results in a coordinated antioxidant and anti-inflammatory response in which phase II detoxification enzymes are activated. Nrf2 regulates GPx, GST, SOD, and TR (all discussed above) as well as glucose-6-phosphate (G6PD)-which provides NADPH to glutathione reductase, heme oxygenase-1 (HO-1)- which generates antioxidant molecules and regulates apoptosis, glutathione reductase (GR)- which catalyzes the reduction of GSSG to GSH, and NAD(P)H:quinone dehydrogenase (NQO1)-a FAD-binding protein [98].

ii.vi. Arsenic-induced mitochondrial toxicity

Arsenic, a recognized cardiovascular toxicant, has exhibited mito-toxic effects in vascular smooth muscle [57], myocardial cells [58], and vascular

endothelial cells [59]. Arsenic is associated with mitochondrial dysfunction through mechanisms such as elevated ROS, induction of apoptosis, and Ca^{2+} overload. In the heart, arsenic causes QT prolongation by altering L-type calcium channels [60]. As previously noted, GSH is also consumed during metabolism of arsenic and in response to arsenic-induced ROS. Indeed, GST utilizes GSH as a substrate and participates in the xenobiotic metabolism of arsenic and other compounds [88] and GPx catalyzes the reduction of H_2O_2 to H_2O with GSH as a substrate [88,100]. Furthermore, decreased availability of GSH due to arsenic toxicity further contributes to ROS accumulation [101].

The pathological effects of ROS are widespread for the cell. For example, lipid peroxidation is a common feature of oxidative stress that has also been attributed to arsenic exposure. In this process, free radicals cause cell damage by sequestering electrons from the lipids in cell membranes [102]. Additionally, arsenic may decrease membrane-based enzyme activity through the generation of ROS [88]. For instance, ROS inhibit the activity of Na^+/K^+ ATPase resting membrane homeostasis, which is critical for maintaining mitochondrial transmembrane potential [103].

In light of arsenic's demonstrated cardiovascular toxicity and the association between mitochondrial dysfunction and ROS, the present study explores

mechanisms of arsenic toxicity and the protective action of food-based antioxidants, many of which work by scavenging antioxidants. We hypothesize that studies provide sufficient proof-of-concept that antioxidant formulations can protect against arsenic-induced mitochondrial dysfunction in cardiovascular cells and tissues.

iii. Methods

We conducted a literature search by screening MEDLINE, Web of Science, and Google Scholar for the terms “mitochondria” “mitochondrial” “toxicity” “toxic” in combination with “heart” “cardiac” “cardiovascular” “myocardial” “myocardium” “arsenic” “ATO” or “arsenic trioxide” and “antioxidant.” We limited our results by eliminating reviews and publications that were not available in English. We had two primary objectives. The first was to test the hypothesis that non-enzymatic antioxidants are highly effective against arsenic-induced cardiovascular toxicity, and may be employed to protect mitochondria in cardiovascular cells and tissues. The second objective of this review was to identify a series of food-based antioxidants that could be supplemented to the diet of arsenic exposed individuals to improve cardiovascular heath, and/or

developed into commercial formulations to include in clinical trials involving arsenic trioxide.

Appropriate hits were analyzed in detail and reference lists were screened for additional appropriate studies. Our initial search returned 308 potential studies to include. One hundred and nineteen studies were eliminated because they were focused on the effects of arsenic on cancer cells, 99 focused on non-cardiovascular cell or tissue types (10 blood, 37 liver/kidney, 16 brain and nervous system, 8 reproductive system, and 28 other), 60 did not investigate the restorative effects of a non-enzymatic antioxidant, 3 had an environmental focus, and 2 were epidemiological studies (**Figure 1**). The remaining 25 articles were included in the present review. We assessed the quality of the studies analyzed in this review in terms of clarity of purpose, experimental design, appropriateness of statistical methods used, thoroughness of data reporting, and impact of primary research article and journal in which the article was published. No additional studies were collected from Web of Science or Google Scholar. We included animal model investigations and cell culture studies in this review. Our search yielded studies investigating isolated heart mitochondria, cardiomyocytes, and cardiovascular tissues.

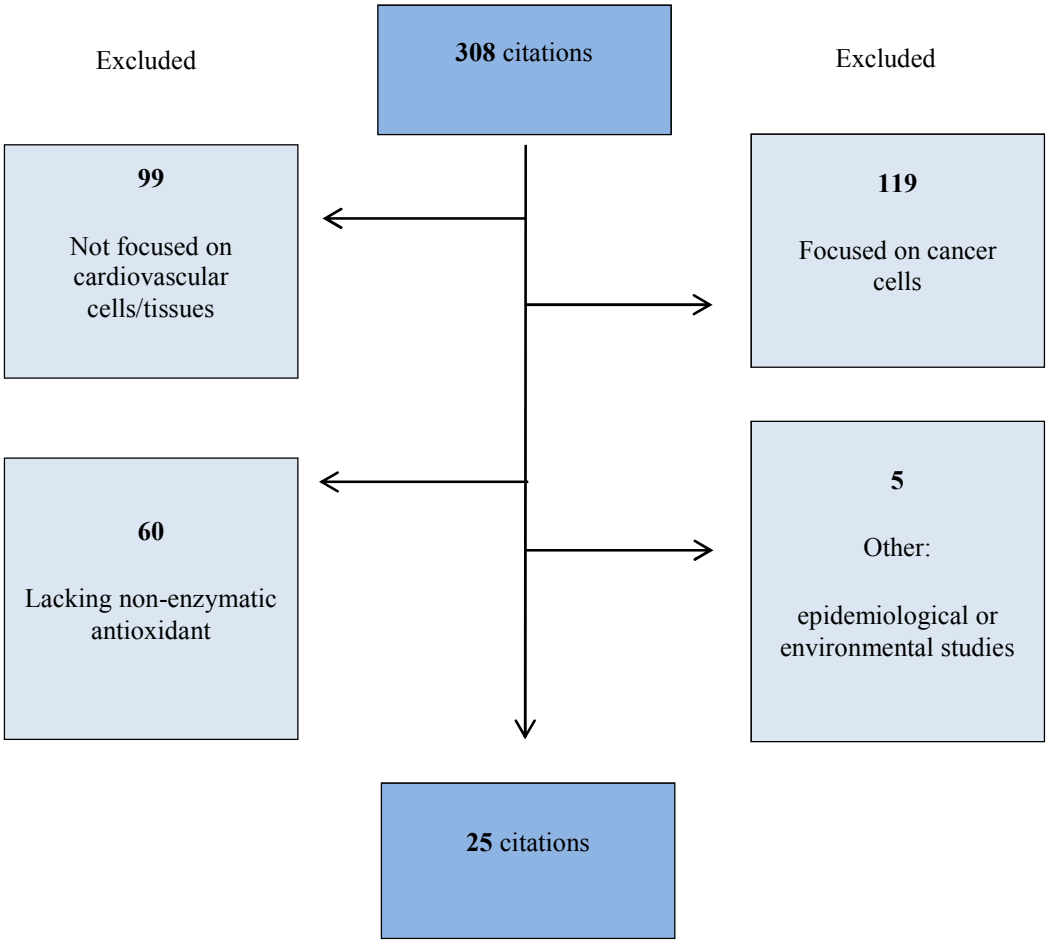


Figure 1: Schematic of selected references

iv. Results

iv.i. Arsenic

iv.i.i. Dose and duration

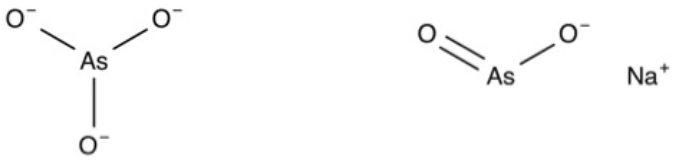
Dose selection justifications varied between studies, but generally, *in vivo* doses were selected based on the literature [89,104-107]. For example, as a fraction of the lethal dose [108], or experimentally as the minimum dose that induced cardio-toxic effects [109]. *In vitro* arsenic doses were selected based on

significant detrimental effects on cell viability and/or cell death and were further stratified by exposure duration (**Table 1**). The studies presented in this review utilized arsenic trioxide (As_2O_3) [58,88,105,106,109-118] or sodium arsenite ($NaAsO_2$) (**Figure 2**) [101,107,108,119-123] at *in vivo* doses ranging from 0.8mg/kg/day to 5mg/kg/day administered intravenously or 2mg/kg/day to 50mg/kg/day administered orally for durations of 6 to 56 days. Experimental animals included Sprague Dawley rats [101,107,122], Wistar rats [111,119], BABL/c mice [124], APO E-/- mice [123], and guinea pigs [110]**.** *In vitro* exposures ranged from 1μM to 20μM arsenic at exposure durations of < 3 to 72 hrs. Cell types included neonatal ventricular cardiomyocytes (NRLVM) [116], rat embryonic cardiomyocytes (H9c2) [114], primary cardiomyocytes isolated from Albino Wistar rats [121], and primary guinea pig cardiomyocytes [110]. Although not the focus of the present review, one study also utilized human promyelotic leukemia (NB4) cells [117].

Table 1. *In vivo* and *in vitro* study design

***In vivo* studies stratified by arsenic exposure duration**					
Duration	**Arsenic Dose**	**Type**	**Method**	**Model**	**Citation**
2 hours	0.15μM, 1.5μM, 5μM	As_2O_3	IV[1]	Wistar rat	[111]
	1.5μM	As_2O_3	IV	Guinea pig	[110]
6-10 days	0.8 mg/kg	As_2O_3	IV	Wistar rat	[116]
	1 mg/kg	As_2O_3	IV	Balb/c mouse	[124]
	3 mg/kg	As_2O_3	IV	Wistar rat	[89]
	10 mg/kg	$NaAsO_2$	Oral	Wistar rat	[120]
	10 mg/kg	$NaAsO_2$	Oral	Wistar rat	[119]
10-29 days	1 mg/kg	As_2O_3	IV	Balb/c mouse	[114]
	2.5 mg/kg	As_2O_3	IP[2]	Wistar rat	[115]
	5 mg/kg	As_2O_3	IP	Wistar rat	[106]
	5 mg/kg	As_2O_3	Oral	SD[3] rat	[122]
	5 mg/kg	$NaAsO_2$	Oral	Wistar rat	[108]
	200 ppb	$NaAsO_2$	Oral	APO E-/-	[123]
30-56 days	2 mg/kg	$NaAsO_2$	Oral	SD rat	[121]
	4 mg/kg	As_2O_3	Oral intubation	Wistar rat	[109]
	4 mg/kg	As_2O_3	Oral intubation	Wistar rat	[109]
	4 mg/kg	As_2O_3	Oral	Wistar rat	[88]
	5 mg/kg	As_2O_3	IV	SD rat	[111]
	10 mg/kg	$NaAsO_2$	Oral	SD rat	[107]
	50 mg/kg	$NaAsO_2$	Oral	SD rat	[101]
***In vitro* studies**					
	1μM	$NaAsO_2$		H9c2[4]	[101]
	1μM, 2μM	As_2O_3		H9c2	[112]
	2μM/mL	As_2O_3		H9c2	[113]
	4μM	As_2O_3		H9c2	[114]
	5μM	As_2O_3		NRLVM[5]	[117]
	5μM	As_2O_3		H9c2	[118]
	5μM	As_2O_3		H9c2	[58]
	5μM	$NaAsO_2$		Primary myocytes	[121]
	5μM, 7.5μM, 10μM	As_2O_3		H9c2	[125]
	5μM, 6μM, 12μM	As_2O_3		NRLVM	[116]
	10μM	As_2O_3		H9c2	[109]
	10μM	$NaAsO_2$		H9c2	[124]

[1]intravenous, [2]intraperitoneal, [3]Sprague Dawley, [4]H9c2-rat heart cardiomyocyte cells,
[5] neonatal rat left ventricular myocytes



Arsenic Trioxide Sodium Arsenite

Figure 2. Arsenic species

iv.i.ii. Cardiovascular structure and function

In vivo, increased arsenic exposure increased arsenic deposition in the heart [105], caused QT interval prolongation [122], increased QTc interval [106], induced ST-T wave change [114], prolonged PQ interval [111], increased RR and QRS interval [122], inhibited I_{KS} current [110], reduced I_K amperage [110] decreased heart rate [122], decreased cardiac output [116], and decreased 3′-5′-cyclic adenosine monophosphate (cAMP), an intracellular messenger that mediates catecholaminergic control of heart rate and contractility [126]. Arsenic exposure increased triglycerides [107], total cholesterol [119], and LDL cholesterol [122], and decreased HDL cholesterol in blood plasma [120]. Arsenic decreased phospholipids, increased free fatty acids (FFA) [108], and increased lipase activity in heart tissues [115]. Arsenic also increased atherosclerotic plaque formation in the aortic arch and aortic sinus [123], and decreased atherosclerotic plaque stability by increasing macrophage content [123].

Arsenic significantly elevated Creatine Kinase (CK) [114] and Creatine Phosphokinase (CPK) [120], which indicate stress to the heart, and elevated Creatine Kinase from muscle and brain (CK-MB) [122], as well as levels of serum troponin [106], which are cardiac markers used to diagnose acute myocardial infarction. Arsenic increased inflammation, measured with C-reactive protein (CRP) [115], and increased the activities of cardiac enzymes lactate dehydrogenase (LDH) [107,122], aminotransferase (AST) [108], alkaline phosphatase (ALP) [108,122], and aspartate aminotransferase (ALT) [108,122], all of which indicate necrotic damage to the heart [127]. Furthermore, arsenic caused notable changes in cardiac structures including cytoplasmic vacuolization, myofibrillar loss, and cardiomyocyte necrosis [124].

iv.i.iii. Cardiac Nrf2

Several *in vivo* studies reported downregulation of cardiac Nrf2 following arsenic exposures ranging from 8-28 days [122], as well as upregulation of Keap-1 [108]. However, Hu et al. (2016) reported an increase in Nrf2 activity after exposing H9c2 cardiomyocytes to 2μM/ml arsenic for 24-hours. Based on arsenic's affinity for sulfhydryls, we expected to see arsenic-induced Nrf2 activation, as reported by past studies on other cell types [128-132]. These

apparently contradictory results suggest a short term upregulation of the Nrf2 pathway in which Nrf2 is modestly activated because cardiomyocytes have a weak ability to excrete arsenic into the extracellular space [133]. This is presumably followed by a more permanent repression of Nrf2 [133].

Since Nrf2 activation stimulates expression of phase II detoxification enzymes, Nrf2 repression should decrease the innate antioxidant response. Indeed, arsenic decreased the activity of enzymes in the Nrf2 pathway including SOD [122], TR [58], GPx [119], GR [108], GST [119], G6PD [108], and hemeoxygenase 1 (HO-1) [122]. Again Hu et al. (2016) had contradictory findings, reporting elevated HO-1 expression, and an increase in NQO1 that likely resulted from short-term elevation in Nrf2.

Arsenic exposure decreased catalase activity [119], decreased GSH levels [120], and decreased GSSG levels and/or GSH/GSSG ratio [119]. In one study, GSH level was elevated at 6 hours following 5μM arsenic exposure [118], but this trend reversed at 12 and 24 hours [118], a result that is consistent with other studies. This observation suggests that arsenic may induce a short-term, compensatory but unsustainable increase in GSH activity. As previously mentioned, GSH is used in the biotransformation of inorganic arsenic to methylated arsenic species, and a temporary increase in GSH may suggest a short-term effect related to arsenic metabolism [118].

iv.i.iv. ROS

In addition to measuring antioxidant activity, many studies have reported ROS levels induced by arsenic exposure. Indeed, cells exposed to arsenic demonstrated an elevation of several ROS and oxidants including H_2O_2 [112,115], mitochondrial superoxide [101,112], ONOO-, and OH- [122]. Arsenic increased lipid peroxidation -measured as malondialdehyde (MDA) [88]- and increased thiobarbituric acid reactive substances (TBARS), a byproduct of lipid peroxidation [119]. Arsenic also increased xanthine oxidase (XO) [118] and increased NOX enzyme activity-an important source of ROS [108]. Interestingly, one study detected a significant increase in nitric oxide (NO) following exposing rats to 5mg/kg/day sodium arsenite for 28 days [122], and one study found that NO changes were not significant following 3mg/kg arsenic exposure in mice on 4 alternate days over an 8-day period [89]. NO is a second messenger involved in the process of blood vessel vasorelaxation, and decreased NO in endothelial cells is associated with elevated blood pressure. Paradoxically, low dose arsenic exposure has been shown to increase NO in endothelial cells, whereas higher dose arsenic exposure decreases NO [134]. Thus, reports of increased NO in response to arsenic exposure likely result from arsenic's non-linear effects based on exposure dose and duration.

iv.i.v. Apoptosis

As expected, arsenic elevated lactate dehydrogenase (LDH) release at various doses and exposure times [89,101,124] and consistently decreased cell viability [112]. Apoptosis was detected via DCFH-DA fluorescence, flow cytometry profile, physiological characteristics (shrinkage, blebbing and rounding up), DNA fragmentation, and/or increased micronuclei frequency [122]. Arsenic also decreased cell growth via DNA synthesis [112].

Arsenic increased caspase-3 cleavage [101], activated caspase-3 [112], caspase-8 [113], and caspase-9 [114], and increased cytochrome c levels in the cytosol [121]. Arsenic increased the level of proteolysis of poly (ADP-ribose) polymerase (PARP) (downstream of caspase-3 activation) [121], increased expression of pro-apoptotic proteins BAX [112], Bad [121], and p53 upregulated modulator of apoptosis (PUMA) [112], and decreased expression of anti-apoptotic proteins Bcl-2, Bcl-xL [112], Survivin [112], X-linked inhibitor of apoptosis protein (XIAP) [112], cellular inhibitor of apoptosis protein-1 (CIAP1), and the ratio of phosphorylated to total anti-apoptotic kinase (AKT) [114]. Arsenic also decreased Bcl-2/BAX protein ratio in one study [114]. Arsenic up regulated TGF-β and Smad3 [122], which both indicate apoptosis (TGF-β triggers Smad3 activation). Additionally, arsenic phosphorylated the nuclear factor-

kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [121]. When phosphorylated, NF-κB translocates to the nucleus and induces the transcription of anti apoptotic proteins such as XIAP1 and Survivin, thus, decreased NF-κB activity has a pro-apoptotic effect. Interestingly, one study reported decreased activity of NF-κB, which is generally considered an anti-apoptotic event [112]. This contradiction may stem from the fact that NF-κB has both inflamatory and anti-inflamatory properties, and apoptosis is an essential mechanism that prevents prolonged inflammation. Thus, increased and decreased NF-κB may reflect different states of cellular stress [135].

It is also worth noting that although a majority of studies have reported on arsenic's ability to induce apoptosis in various cell types including aortic, coronary, and mesenteric smooth muscle cells [136,137], several past studies have also reported that exposure to inorganic arsenic results in necrosis whereas the metabolite monomethylarsonous acid (MMA) primarily results in caspase-dependent apoptosis in vascular smooth muscle cells [57] and other cell types [138-140]. Variations in the method of cell death may result from differences in the arsenic dose, duration, and cell type (vascular smooth muscle vs. cardiomyocytes). In the present review, we report that among cardiomyocytes, inorganic arsenic promotes ROS mediated apoptosis.

iv.i.vi. Calcium overload

Arsenic increased Ca^{2+} content [88], increased Cav 1.2 protein expression [116], and decreased Ca-ATPase [88]. Calcium overload results in collapsed mitochondrial transmembrane potential. Indeed, arsenic consistently decreased mitochondrial transmembrane potential [116], a sign of decreased mitochondrial health. Arsenic also decreased Na^{+}/K^{+} ATPase activity, which regulates mitochondrial transmembrane potential [88,108], and elevated phosphorylation of JNK and p-38 MAPK, which mediate mitochondrial membrane collapse [116].

iv.i.vii. Mitochondrial function

Arsenic decreased the activity of mitochondrial complexes I [58], II [122], III [58], and IV [58], decreased steady-state levels of ATP [58], decreased Mg^{2+}ATPase, [108], and increased the level of mitochondrial superoxide [112]. Arsenic caused mitochondrial swelling, mitochondrial pore opening [58], and other alterations in mitochondrial morphology [122]. In one study, arsenic decreased the activities of heart mitochondrial enzymes including isocitrate dehydrogenase (ICDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH), and NADH dehydrogenase [108]. Arsenic also decreased the activity of aconitase, an indicator of increased mitochondrial superoxide and essential

enzyme in the citric acid cycle that converts citrate into iso-citrate [141]. Arsenic increased autophagy in cardiomyocytes, as measured by assaying for the level of the autophagic markers microtubule-associated protein 1A/1B-light chain 3-phosphatidylethanolamine conjugate (LC3-II)/ microtubule-associated protein 1A/1B-light chain 3 (LC3-I) [117]. In the process of autophagy, LC3 is conjugated with phosphatidylethanolamine to form LC3- phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes in autophagic vacuoles. The autophagosomes subsequently fuse with lysosomes to form autolysosomes, thus contributing to autophagic cell death [142].

This data is consistent with previous studies showing that arsenic induces widespread mitochondrial dysfunction via loss of mitochondrial membrane potential, generation of ROS, diminution of cytochrome-C oxidase function, ROS-dependent activation of autophagy, and suppression of oxygen consumption via depletion of mtDNA copy number [143,144]. In addition, a recent study found that inorganic arsenic and/or methylated arsenicals decrease ATP content, increase the level of hydrogen peroxide and mitochondrial superoxide, cause aberrant nuclear clustering of mitochondria and decrease mitochondrial content in vascular smooth muscle cells [57].

The studies reviewed here clearly demonstrate that arsenic impacts all aspects of mitochondrial function including energy production, calcium storage, ROS, and activities of mitochondrial-regulated cell death signaling pathways.

iv.ii. Antioxidants

The studies selected for this review analyzed the abilities of antioxidants to counteract the toxic effects of arsenic on mitochondria in cardiovascular tissues and cells (**Table 3**). It is worth noting that the majority of these antioxidants were phenolic compounds. Polyphenolic compounds belong to a heterogeneous group of chemicals that contain one or more aromatic rings and one or more hydroxyl groups. Polyphenols have non-enzymatic antioxidant activities that scavenge free radicals and oxidants such as $O^{\bullet-}$, $ONOO^-$ and H_2O_2. Oxidants are deactivated by polyphenolic antioxidant (POH) donation of a hydrogen atom, forming a phenoxyl radical ($PO^{\bullet}$), which is then stabilized through intermolecular bonding between two polyphenols (**Figure 3**).

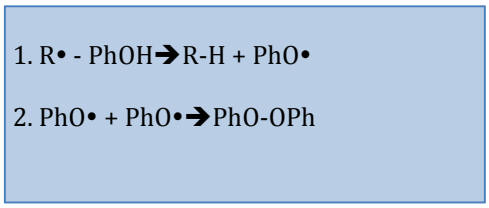


Figure 3: Polyphenolic neutralization of ROS

Polyphenols can be subcategorized as flavonoids, stilbenes, phenolic acids, and lignins (**Table 2**). Flavonoids are subcategorized as isoflavones, flavonols, flavones, flavanols, proanthocyanidins, and anthocyanins [145].

Table 2. Antioxidant classification and source

Polyphenolic Antioxidants	Classification	Source
Biochanin A [107]	Flavonoid	Cabbage, alfalfa
Boerhavia diffusa [125]	Flavonoid	*B. diffusa*
Ellagic acid [106]	Phenol	Berries, walnuts
EGCG [101]	Catechin	Green tea
Eugenol [88]	Phenol	Clove
Genistein [117]	Flavonoid	Soy
Grape seed and skin extract [115]	Flavonoid, stilbene	Grapes
Imperatorin [113]	Flavonoid	*Radix Saposhininkovaie*
Sec-o-glucosylhamandol [113]	Flavonoid	*Radix Saposhininkovaie*
Malus domestica apple peel [118]	Flavonoid	Apples
Naringin [122]	Flavonoid	Citrus fruit
Phloretin [58]	Flavonoid	Apples
Resveratrol [89]	Stilbene	Red wine
Silybum marianum [108]	Flavonoid	Milk thistle
Sorbus phnuashanesis (Hante) Hedl[114	Flavonoid	Chinese herb
Trichosanthes dioca [120]	Flavonoid	*T. dioica*
Other antioxidants	Type	Source
α-lipoic acid [111]	Organosulfur compound	Spinach, broccoli
Flax seed oil [105]	α-linoleic acid	Flax seeds
Morphine [112]	Opioid	Poppy seeds
Omega-3 fatty acid [109]	Polyunsaturated fatty acid	Fish oil
Selenium [123]	Essential trace element	Lentils
Taurine [121]	Sulfonic acid	Amino acid cysteine

Table 3. Arsenic induced effects and antioxidant restoration

Arsenic induced effect	Restored with antioxidant	Citation
	Heart	
Arsenic deposition in heart	Eugenol	[88]
	Grape seed & skin extract	[115]
	EGCG	[101]
	Omega-3	[109]
	Flax seed oil	[105]
	Resveratrol	[89]
QT interval prolongation	Naringin	[122]
	Eugenol	[88]
	Genistein	[116]
	α-lipoic acid	[110]
	Sorbus phnuashanesis	[114]
	Resveratrol	[124]
Increased QTc interval	Naringin	[122]
	Ellagic acid	[106]
	α-lipoic acid	[110]
ST-T wave change	Sorbus phnuashanesis	[114]
Increased RR interval	Naringin	[122]
Increased QRS interval	Naringin	[122]
Inhibited I_{KS} currents	α-lipoic acid	[110]
Reduced amperage of I_K	α-lipoic acid	[110]
Decreased heart rate	Naringin	[122]
	Eugenol	[88]
	Genistein	[116]
Decreased cardiac output	Genistein	[116]
Decreased CAMP	Resveratrol	[89]
Structural changes in cardiac tissue	Naringin	[122]
	Eugenol	[88]
	Ellagic acid	[106]
	Grape seed & skin extract[1]	[115]
	EGCG	[101]
	Omega-3	[109]
	Flax seed oil	[105]
	Sorbus phnuashanesis	[114]
	Resveratrol[1]	[89]
	Resveratrol[1]	[124]
Atherosclerotic plaque formation	Selenium	[123]
Increased ALP activity	Naringin	[122]
	Silybum marianum	[108]
	Blood Plasma	
Elevated triglycerides	Biochanin A	[107]
	Grape seed & skin extract	[115]

Increased total cholesterol	Naringin	[122]
	T. dioica root	[120]
	T. dioica fruit	[119]
	Taurine	[121]
	Silybum marianum	[108]
	Grape seed & skin extract	[115]
Increased LDL cholesterol	naringin	[122]
	Biochanin A	[107]
	Silybum marianum	[108]
Decreased HDL cholesterol	*T. dioica* root	[120]
	T. dioica fruit	[119]
Decreased phospholipids	*Silybum marianum*	[108]
Increased atherogenic Coefficient (AC)	Biochanin A	[107]
Increased cardiac risk ratio	Biochanin A	[107]
Increased free fatty acids	*Silybum marianum*	[108]
Increased lipase activity	GSSE	[115]
Increased CPK	*T. dioica* root	[120]
	T. dioica fruit	[119]
Increased CK-MB	Naringin	[122]
	Eugenol	[88]
	Ellagic Acid	[106]
	Silybum marianum	[108]
	EGCG	[101]
	Omega 3	[109]
	Flax seed oil	[105]
	Sorbus phnuashanesis	[114]
	Resveratrol	[89]
Increased serum troponin	Ellagic acid	[106]
Elevated LDH	Naringin	[122]
	T. dioica root	[120]
	T. dioica fruit	[119]
	Eugenol	[88]
	Biochanin A	[107]
	Silybum marianum	[108]
	EGCG	[101]
	Flax seed oil	[105]
	Sorbus phnuashanesis	[114]
	Resveratrol	[89]
	Resveratrol	[124]
Increased AST activity	Naringin	[122]
	α-lipoic acid	[111]
	Silybum marianum	[108]
	EGCG	[101]
	Resveratrol	[89]
Increased ALT activity	Naringin	[122]

	Silybum marianum	[108]
Increased ALP activity	*Silybum marianum*	[108]
Increased CRP	Grape seed & skin extract	[115]
Increased CK	Sorbus phnuashanesis	[114]
	Resveratrol	[89]
	Antioxidants	
Downregulated NRf2	Naringin	[122]
	Silybum marianum (liver)	[108]
	Sorbus phnuashanesis	[114]
	Resveratrol	[89]
Increased Nrf2 level	sec-O-glucosylhamaudol[2]	[113]
	Imperatorin[2]	[113]
Upregulation of Keap-1	*Silybum marianum* (liver)	[108]
Decreased SOD activity	Naringin	[122]
	T. dioica root	[120]
	T. dioica fruit	[119]
	Resveratrol	[117]
	Genistein	[117]
	Taurine	[121]
	Biochanin A	[107]
	Silybum marianum	[108]
	Grape seed & skin extract	[115]
	EGCG	[101]
	Omega-3	[109]
	Flax seed oil	[105]
	Boerhavia diffusa	[125]
	Malus domestica L. Peel	[118]
	Phloretin	[58]
	Sorbus phnuashanesis	[114]
	Resveratrol	[124]
Decreased TR activity	Phloretin	[58]
Decreased GPx activity	*T. dioica* root	[120]
	T. dioica fruit	[119]
	Eugenol	[88]
	Taurine	[121]
	Silybum marianum	[108]
	Grape seed & skin extract	[115]
	EGCG	[101]
	Omega-3	[109]
	Flax seed oil	[105]
	Malus domestica L. Peel	[118]
	Phloretin	[58]
	Sorbus phnuashanesis	[114]
	Resveratrol	[124]
Increased GPx activity	Ellagic acid	[106]
Decreased GR activity	*T. dioica* root	[120]

	T. dioica fruit	[119]
	Taurine	[121]
	Silybum marianum	[108]
	Malus domestica L. Peel	[118]
Decreased GST activity	*T. dioica* root	[120]
	T. dioica fruit	[119]
	Eugenol	[88]
	Taurine	[121]
	Silybum marianum	[108]
	Omega-3	[109]
	Flax seed oil	[105]
Decreased G6PD activity	*Silybum marianum*	[108]
Downregulated HO-1	Naringin	[122]
	Silybum marianum (liver)	[108]
	Sorbus phnuashanesis	[114]
	Resveratrol	[89]
Elevated HO-1 expression	Imperatorin	[113]
	Sec-O-glucosylhamaudol[2]	[113]
Elevated NQ01 expression	Imperatorin	[113]
	Sec-O-glucosylhamaudol	[113]
Decreased catalase activity	*T. dioica* root	[120]
	T. dioica fruit	[119]
	Taurine	[121]
	Biochanin A[3]	[107]
	Silybum marianum	[108]
	Grape seed & skin extract	[115]
	Omega-3	[109]
	Flax seed oil	[105]
	Boerhavia diffusa	[125]
	Malus domestica L. Peel	[118]
	Sorbus phnuashanesis	[114]
	Resveratrol	[124]
Decreased GSH levels	Naringin	[122]
	T. dioica root	[120]
	T. dioica fruit	[119]
	Eugenol	[88]
	Resveratrol	[117]
	Taurine	[121]
	Biochanin A[c]	[107]
	Silybum marianum	[108]
	Omega-3	[109]
	Flax seed oil	[105]
	Phloretin	[58]
No significant change in GSH	Ellagic acid	[106]
Short term GSH elevation followed by decrease	Malus domestica L. Peel	[118]

Increased GSSG	Selenium	[123]
	T. dioica root	[120]
	T. dioica fruit	[119]
Decreased GSH/GSSG ratio	Taurine	[121]
	Resveratrol	[89]
	ROS	
Elevated H_2O_2	Grape seed & skin extract	[115]
Elevated mitochondrial ROS	Morphine	[112]
	EGCG	[101]
	Malus domestica L. Peel	[118]
	Phloretin	[58]
Elevated (H_2O_2, $ONOO^-$, OH^-)	Naringin	[122]
	Morphine	[112]
	Resveratrol	[117]
	Genistein	[117]
	Imperatorin	[113]
	Phloretin	[58]
	Sorbus phnuashanesis	[114]
	Boerhavia diffusa	[125]
	Malus domestica L. Peel	[118]
	Resveratrol	[89]
	Resveratrol	[124]
Lipid peroxidation (elevated MDA)	Naringin	[122]
	Eugenol	[88]
	Ellagic acid	[106]
	Biochanin A^3	[107]
	Grape seed & skin extract	[115]
	EGCG	[101]
	Taurine	[121]
	Omega-3	[109]
Increased 8-OHdG	α-lipoic acid	[111]
	Resveratrol	[89]
Elevated TBARS	*T. dioica* root	[120]
	T. dioica fruit	[119]
	Silybum marianum	[108]
	Flax seed oil	[105]
Increased XO	Malus domestica L. Peel	[118]
	Phloretin	[58]
Increased NOX activity (NOX2 and NOX4)	*Silybum marianum*	[108]
No change in NO	Resveratrol	[89]
Increased NO content	Naringin	[122]
	Apoptosis	
LDH release	Taurine	[121]
	Imperatorin	[113]
	Sec-O-glucosylhamaudol	[113]

	EGCG	[101]
	Omega-3	[109]
	Boerhavia diffusa	[125]
	Malus domestica L. Peel	[118]
	Phloretin	[58]
	Sorbus phnuashanesis	[114]
	Resveratrol	[89]
	Resveratrol	[124]
Decreased cell viability	Morphine	[112]
	Resveratrol	[117]
	Genistein	[117]
	Taurine	[121]
	Imperatorin	[113]
	Sec-O-glucosylhamaudol	[113]
	EGCG	[101]
	Boerhavia diffusa	[125]
	Malus domestica L. Peel	[118]
	Phloretin	[58]
	Sorbus phnuashanesis	[114]
	Resveratrol	[124]
Apoptosis	Naringin	[122]
	Genistein	[116]
	Resveratrol + Genistein	[117]
	Taurine	[121]
	Imperatorin	[113]
	Sec-O-glucosylhamaudol	[113]
	EGCG	[101]
	Omega-3	[109]
	Boerhavia diffusa	[125]
	Malus domestica L. Peel	[118]
	Phloretin	[58]
	Sorbus phnuashanesis	[114]
	Resveratrol	[124]
DNA fragmentation	Morphine	[112]
	T. dioica root	[120]
	T. dioica fruit	[119]
	Genistein	[117]
	Silybum marianum	[108]
	Omega 3	[89]
	Resveratrol	[124]
Decreased cell growth via DNA synthesis	Morphine	[112]
Increased caspase-3 cleavage	Genistein	[117]
	EGCG	[101]
Elevated caspase-3 activity	morphine	[112]
	Genistein	[116]

	Taurine	[121]
	Imperatorin[3]	[113]
	Sec-O-glucosylhamaudol[3]	[113]
	EGCG	[101]
	Sorbus phnuashanesis	[114]
	Malus domestica L. Peel	[118]
	Phloretin	[58]
	Resveratrol	[124]
Elevated caspase-8 activity	Sorbus phnuashanesis	[114]
Elevated caspase-9 activity	Sorbus phnuashanesis	[114]
Elevated cytochrome-c	Taurine	[121]
Proteolysis of PARP	Taurine	[121]
Increased BAX	Morphine	[112]
	Taurine	[121]
Increased Bad	Taurine	[121]
Increased PUMA	Morphine	[112]
Decreased Bcl_2	Morphine	[112]
	Taurine	[121]
Decreased Bcl-xL	Morphine[3]	[112]
	Taurine	[121]
Decreased Bcl2/BAX ratio	Sorbus phnuashanesis	[114]
Decreased CIAP1, CIAP2, XIAP	Morphine[3]	[112]
Decreased Survivin	Morphine	[112]
Decreased P-Akt/Akt	Sorbus phnuashanesis	[114]
Upregulated TGF-β	Naringin	[122]
Increased SMAD3	Naringin	[122]
Calcium and Membrane potential		
Decreased NF-κB activity	Morphine	[112]
Phosphorylated NF-κB	Taurine	[121]
Calcium accumulation	Eugenol	[88]
	Genistein	[116]
	Taurine	[121]
	Imperatorin[3]	[113]
	Sec-O-glucosylhamaudol[3]	[113]
	Grape seed & skin extract	[115]
	EGCG	[101]
	Omega-3	[109]
	Boerhavia diffusa	[125]
	Malus domestica L. Peel	[118]
	Phloretin	[58]
	Resveratrol	[124]
	Resveratrol	[89]
Increased in Cav1.2	Genistein	[116]
Decreased Ca-ATPase activity	Eugenol	[88]
	Silybum marianum	[108]

	Phloretin	[58]
Decreased Na^+/K^+ ATPase activity	Naringin	[122]
	Eugenol	[88]
	Silybum marianum	[108]
Decreased transmembrane potential	Resveratrol	[117]
	Genistein	[117]
	Taurine	[121]
	Genistein	[116]
	Omega-3	[109]
	Phloretin	[58]
	Boerhavia diffusa	[125]
Elevated phosphorylation of JNK and p-38 MAPK	Genistein	[116]
	Taurine	[121]
	Mitochondria	
Decreased activity at mito. complex I, III, IV	Naringin	[122]
	Phloretin	[58]
Decreased activity at mito. complex II	Naringin	[122]
	Morphine	[112]
Decreased ATP content	Phloretin	[58]
Decreased Mg^{2+} ATPase	*Silybum marianum*	[108]
Altered mitochondrial morphology	Naringin	[122]
	Silybum marianum	[108]
	Phloretin	[58]
Decreased OCR	Phloretin	[58]
Mito. swelling and pore opening	Phloretin	[58]
Decreased activities of heart mitochondrial enzymes	*Silybum marianum*	[108]
Decreased aconitase activity	Phloretin	[58]
Increased LC3-II/LC-31	Genistein[2]	[117]
	Resveratrol[2]	[117]

[1] partially restored, [2] further increased, [3] not restored

Several studies investigated non-polyphenolic compounds. These included taurine, a sulfonic acid derivative of the amino acid cysteine; omega-3 fatty acid, which is found in fish oil and contains eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); flax seed oil, an alpha-linoleic acid; and the organosulfur compound alpha-lipoic acid.

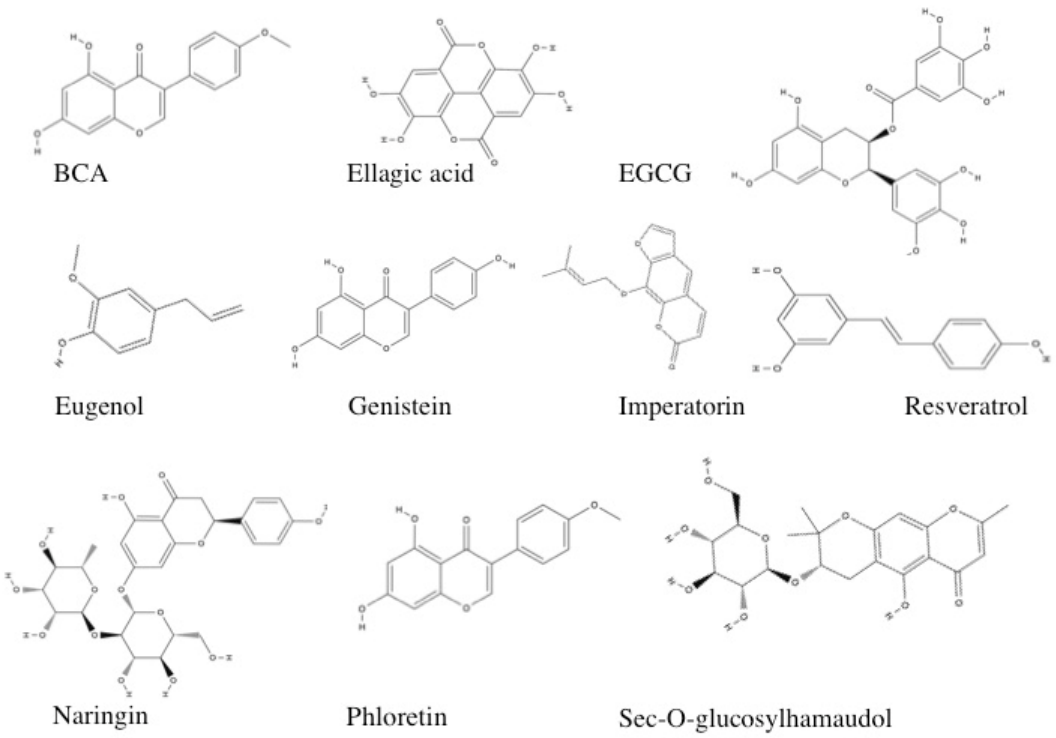


Figure 4. Polyphenolic antioxidant structures

iv.ii.i. Polyphenols (**Figure 4**)

- Biochanin A

Biochanin A (BCA) is found in red clover, cabbage, and alfalfa. BCA is a flavonoid polyphenol that scavenges free radicals and chelates and mobilizes toxins due to the presence of methoxy substitutions [107,146]. BCA is initially metabolized into genistein, but if excess BCA is available it is metabolized into both genistein and daidzein. Although daidzein is an isoflavone, it has relatively

poor antioxidant properties and can induce oxidative stress by generating free radicals [107].

Sprague Dawley rats were orally treated with sodium arsenite at a concentration of 10mg/kg/day or co-administered arsenic with BCA at 10, 20, or 40mg/kg/day for 6 weeks [107]. BCA was unable to restore arsenic-induced elevations in MDA [107]. BCA at the 20μM concentration significantly reduced SOD but was incapable of restoring GSH and catalase in heart tissue [107].

BCA significantly restored triglyceride levels, LDL and V-LDL cholesterol, atherogenic coefficient (AC)- a ratio of non-HDL cholesterol to HDL cholesterol, and cardiac risk ratio (CRR)- a ratio of non-HDL cholesterol to total cholesterol [107]. No pathological signs of arsenic toxicity were detected in heart tissues [107]. It is worth noting that although BCA was not effective at restoring all parameters, BCA alone had no negative effects on any variables in normal rats. The authors observed that BCA was only moderately effective at restoring arsenic-induced damages in cardiovascular tissues because of the pro-oxidative properties of the metabolite daidzein [107].

Although not the focus of the present study, BCA at 20mg/kg and 40mg/kg restored renal dysfunction, measured with urinary urea, creatinine, and BUN ratio. BCA at 20mg/kg reduced MDA, GSH content, SOD activity, and catalase

activity in kidney tissues and protected kidney architecture compared to arsenic-exposed tissue [107].

- *Boerhavia diffusa*

Boerhavia diffusa (BDE) is a potent antioxidant that contains biologically active polyphenols including retinoids and flavonoids as well as amino acids, lignins, saponins, b-sitosterols, and tetracosanoic, eicosanoic, stearic, and ursolic acids [125,147].

H9c2 cardiomyocytes were treated with 5μM, 7.5μM, and 10μM arsenic in the presence or absence of 20μg/mL BDE for 24-hours. BDE reversed many of the effects of arsenic in H9c2 cardiomyocytes, particularly at the lower doses of arsenic tested. BDE (20μg/mL) prevented alterations in morphology and cell viability caused by 5μM and 7.5μM arsenic [125]. BDE also increased the uptake of neutral red (NR) a supravital dye that viable cells incorporate in the lysosome- which had been reduced with 5μM arsenic, and significantly rescued LDH release from cells treated with all arsenic concentrations [125]. BDE reduced ROS and oxidants (H_2O_2, OH-, ONOO-), and reduced morphological aberrations of apoptosis caused by exposure to 5μM [125]. BDE restored transmembrane potential and reversed Ca^{2+} influx caused by

exposure to 5μM and 7.5μM arsenic. Additionally BDE reversed alterations in lysosome, ER, and contractile protein morphology in cells exposed to 5μM and 7.5μM arsenic, and maintained ER integrity in cells exposed to 5μM arsenic [125].

- Ellagic acid

Ellagic acid is a phenolic compound found in blackberries, raspberries, strawberries, cranberries, grapes, pomegranate, and walnuts [148]. Ellagic acid is considered a potent antioxidant and exhibits antibacterial, antiviral, anti-inflammatory, anti-fibrotic, anti-atherogenic, anti-mutagenic, and immunoregulatory properties [149]. The authors orally administered 30mg/kg of ellagic acid to Wistar rats, followed one-hour later by an intraperatonial injection of 5mg/kg arsenic for 10 days. Ellagic acid co-treatment increased QTc interval and decreased the cardiac biomarkers troponin-I and CK-MB [106]. Ellagic acid co-treatment significantly decreased GPx activity, and significantly decreased lipid peroxidation in the heart tissue of experimental animals [106]. Ellagic acid also prevented arsenic-mediated myofibrillar loss and myofibrillar coagulative necrosis [106].

- EGCG

(-)-Epigallocatechin-3-gallate (EGCG) is a catechin and the most abundant flavonoid found in green tea [101]. EGCG has potent antioxidative activity because of the two triphenolic groups in its molecular structure [101] and has been used for the treatment of cancer, cardiovascular diseases, and autoimmune disease [150,151].

Sun et al. (2016) exposed Sprague-Dawley rats to sodium arsenite at a concentration of 50mg/kg/day alone or in combination with EGCG at a concentration of 50mg/kg/day for 30 days. Additionally, H9c2 cardiomyocytes were treated with 1μM arsenic alone or with 1μM EGCG for 24 hours.

EGCG fully reversed morphological changes in the myocardium including necrosis, intracellular edema, myofibrillar derangements, swollen and damaged mitochondria, and wavy degeneration of muscle fibers [101]. EGCG significantly reduced arsenic accumulation in the hearts of experimental rats and significantly inhibited arsenic-induced elevations in the activities of the cytoplasmic enzymes LDH, CK-MB, and AST in tissue [101].

EGCG co-treatment significantly reduced apoptosis, significantly increased the catalytic activities of SOD, catalase, and GPx, significantly decreased lipid peroxidation, and restored calcium balance [101]. In H9c2 cells, EGCG co-

treatment significantly decreased LDH release, increased cell viability, and decreased apoptosis, caspase-3 activity, and the level of cleaved caspase-3. Additionally, EGCG significantly inhibited arsenic mediated mitochondrial ROS [101].

- Eugenol

Eugenol is a phenolic monoterpene and member of the allylbenzene chemical class of compounds. Eugenol is extracted from clove and has antioxidant activity attributed to its methoxyphenolic structure [88].

Binu et al. (2017) orally exposed Wistar rats to 4mg/kg arsenic with or without 5mg/kg eugenol for 30 days. Electrocardiograph (ECG) readings performed on anesthetized rats demonstrated that arsenic prolonged QT interval and caused low heart rate, while co-treatment with eugenol significantly corrected these measures [88]. Eugenol co-treatment significantly decreased sodium and calcium tissue electrolytes, and increased potassium tissue electrolytes compared to arsenic exposed rats [88].

Eugenol significantly increased Ca^{2+}-ATPase activity and decreased Na^{+}/K^{+}-ATPase activity compared to the arsenic treated group [88]. Co-treatment with

eugenol also significantly decreased levels of the cardiac markers CK-MB and LDH, and increased GSH content, GST activity, and GPx activity [88].

Lipid peroxidation was significantly elevated in the arsenic treated group, and significantly restored from eugenol co-treatment. Eugenol co-treatment also significantly decreased arsenic deposition in the heart and restored structural abnormalities in the myocardium of arsenic treated rats to near normalcy [88]. Although the mechanism is not fully elucidated, the authors suggest that eugenol may trap free radicals to protect the myocardium from oxidative injury [88].

- Genistein

Genistein is a natural biologically active flavonoid found in soy that has anti-cancer, anti-inflammatory and antioxidant properties [116]. Wistar rats were intravenously administered saline or genistein (10, 50, or 100mg/kg/day) for 7 days, after which arsenic trioxide (0.8 mg/kg/day) was co-administered for another 7 days. Additionally, primary neonatal rat ventricular cells (NRVCs) were exposed to 10, 50 or 100μM genistein for 1-hour followed by incubation with 5μM arsenic for various time points.

Pre-treatment with genistein (10, 50, 100μM) attenuated apoptosis in NVRCs exposed to 5μM arsenic for 24-hours [116]. Genistein also attenuated arsenic-

induced up-regulation of JNK phosphorylation, phosphorylation of 38-MAPK (p38-MAPK), and cleaved caspase-3 *in vivo* and *in vitro* from exposure to 5μM arsenic for 24-hours [116]. In NVRCs, genistein dose-dependently attenuated the collapse of mitochondrial membrane potential induced with 12-hour exposure to 5μM arsenic, and DNA fragmentation induced with 24-hour exposure to 5μM arsenic [116]. In addition, pretreatment with genistein was effective against intracellular Ca^{2+} overload in NVRCs induced with 6-hour exposure to 5μM arsenic, and up-regulation of Cav1.2 (an alpha1 subunit of L-type calcium channel carrying IcaL) *in vivo* and in NVRCs following 6-hour exposure to 5μM arsenic.

In vivo, genistein dose-dependently shortened arsenic-induced QT interval prolongation, and improved cardiac function impairment, including arsenic-induced reduced heart rate and reduced cardiac output in Wistar rats [116].

- Grape seed and skin extract

Grape seed and skin extract (GSSE) is a polyphenolic mixture containing flavonoids, stilbenes, proanthocyanidins, and other polyphenols [115]. The antioxidative effects of GSSG may be due to flavonoid components such as quercetin, which has independently been shown to reduce oxidative stress [152],

resveratrol, a stilbene found in GSSE, or due to a synergism between polyphenols [115].

Wistar rats were exposed to 2.5mg/kg of arsenic trioxide in the presence or absence of 4g/kg GSSE for 21 days. Arsenic did not significantly alter body mass or heart mass of experimental animals, nor were total cholesterol or triglyceride levels affected. Alternately, arsenic exposure did provoke myocardial inflammation (measured with plasma CRP and LDH) and led to significantly elevated arsenic concentrations in plasma and heart tissue, while GSSE co-treatment significantly corrected the above disturbances to near control levels [115]. GSSE counteracted arsenic-induced increases in lipid peroxidation, carbonylation and non-protein sulfhydryl (NPSH) in the heart, all of which are promoted by ROS [115]. GSSG-co treatment also rescued catalase activity, GPx activity, and SOD activity to near control levels.

Interestingly, expression of the Cu/Zn SOD isoform was highly depressed in this study, while the Mn SOD isoform was unchanged. Thus the authors investigated the effect of arsenic on transition metals and determined that arsenic depleted copper in the heart, slightly increased zinc level in the heart, and had no effect on heart manganese [115]. In agreement with these findings, arsenic depressed the copper-dependent enzyme tyrosinase, slightly increased LDH (a

zinc containing enzyme), and had no effect on the manganese-dependent glutamine synthase [115]. Heart copper deficiency is a recognized cause of myocardial fibrosis and heart failure. Copper deficiency has also been linked to cardiomyopathy, decreased cytochrome-c oxidase activity, hydrogen peroxide generation, and calcium dysregulation [153]. The authors suggest that the effect of arsenic on transition metal distribution (i.e. copper depletion and iron overloading), could be an initiation point of arsenic induced oxidative stress [115]. Importantly, GSSE significantly counteracted the effects of arsenic on transition metals and enzyme activities [115].

Compared to arsenic exposed rats, GSSE significantly decreased triglycerides, cholesterol, and lipase activity in the hearts of co-treated rats [115]. GSSE-co treatment also significantly decreased hydrogen peroxide, free iron, ionizable calcium, and calpain activity (a calcium dependent protease) [115]. GSSE was partially effective at correcting arsenic-induced effects on myocardial architecture such as decreased myocardial size, cytoplasmic vacuolization, myofibrillar loss, and mild swelling of fibers with mild interstitial edema [115].

- Imperatorin and sec-O-glucosylhamaudol

Radix Saposhnikoviae is a traditional Chinese medicine from the root of *Saposhnikovia divaricata (Turcz.) Schischk* that contains the flavonoid components imperatorin and sec-O-glucosylhamaudol [113].

These two extracts were compared at a variety of concentrations for their ability to counteract the toxic effects of arsenic trioxide on cardiomyocytes. Hu et al. (2016) administered antioxidants 1-hour prior to exposing cardiomyocytes to 2μM/mL arsenic for 24 hours. Both imperatorin (20, 30, 40 up to 90μg/ml) and sec-O-glucosylhamaudol (60, 70, 80μg/ml) protected against arsenic-induced cell death [113]. Fifty micrograms per milliliter of imperatorin or 50μg/ml sec-O-glucosylhamaudol prevented LDH release. Imperatorin (100μg/ml) and sec-O-glucosylhamaudol (50μg/ml) prevented apoptosis, however, neither antioxidant decreased caspase-3 activity. Imperatorin (50 and 60μg/ml) reduced ROS and oxidants (H_2O_2, HO-, and ONOO-), but sec-O-glucosylhamaudol failed to suppress ROS. Neither imperatorin nor sec-O-glucosylhamaudol significantly restored arsenic induced elevations in cellular calcium levels [113].

Compared to control cells, single exposure to arsenic, imperatorin, or sec-O-glucosylhamaudol increased mRNA expression of Nrf2, NQO1, and HO-1. Pretreatment of cells with imperatorin or sec-O-glucosylhamaudol, followed by

arsenic exposure led to a further significant increase in mRNA levels of Nrf2 compared to arsenic treated cells. Additionally, pre-treatment with imperatorin (100μg/mL) or sec-O-glucosylhamaudol (50μg/mL) followed by arsenic exposure further significantly increased the expression levels of NQO1 mRNA [113]. In contrast, Imperatorin pre-treatment (50μg/mL) significantly decreased HO-1, yet sec-O-glucosylhamaudol had no significant effect on HO-1 in combination with arsenic.

In agreement with the effects of these antioxidants on mRNA expression, co-exposure to arsenic and imperatorin or sec-O-glucosylhamaudol significantly increased NQO1 protein levels. Again, imperatorin (75μg/mL) co-treatment decreased HO-1 protein levels, whereas Sec-O-glucosylhamaudol co-treatment further significantly increased HO-1 protein levels. Although the authors do not offer an explanation for this apparent contradiction, they suggest that future studies should examine the significance of the downregulation of HO-1 mRNA and protein expression [113].

- Malus domestica apple peel extract

Malus domestica L. peel contains polyphenolic flavonoids such as anthocyanins, qurecetin glycosides and cyaniding glycoside [118]. Malus

domestica L. peel has been reported to inhibit arsenic trioxide-induced LDL oxidation, reduce ROS, exhibit antihypertensive properties, and protect against damaged DNA and mitochondria [118]. Vineetha et al. (2014) investigated aqueous and methanolic extract of apple peel (40μg/mL) against 5μM arsenic in H9c2 cells exposed for 24-hours (unless otherwise noted).

Both aqueous extract and methanolic extracts demonstrated high phenolic and flavonoid content [118]. Co-treating H9c2 cardiomyocytes with apple peel extracts effectively increased cell viability, decreased LDH release, and reduced morphological indications of apoptosis induced by arsenic trioxide. Apple peel extracts decreased mitochondrial superoxide, ROS, and oxidants (H_2O_2, OH-, and ONOO-). Apple peel extracts also reversed arsenic induced alterations in GPx activity to near control levels, reversed short term (6-hour) increases in GSH content and longer term (≥12-hour) decreases in GSH content.

Arsenic exposure significantly decreased SOD and catalase activity, while apple peel extracts significantly reversed these trends [118]. Apple peel extracts decreased XO activity, caspase-3 activity, and calcium levels, and increased TR activity to near similar levels as untreated cells [118].

- Naringin

Naringin, a flavonoid antioxidant, is a polyphenolic compound found in citrus fruit that has anti-inflammatory, antioxidant, antihypertensive, and anti-cancer activities [122].

Adil et al. (2016) orally exposed Sprague-Dawley rats to 5mL/kg arsenic for 28 days in the presence or absence of Naringin (20, 40, or 80mg/kg). Naringin (40 and 80mg/kg) increased heart weight, body weight, and heart rate, and decreased QRS, QT, QTc, and RR interval compared to arsenic-only treated rats [122]. Naringin (40 and 80mg/kg) also significantly decreased systolic and diastolic blood pressures, and significantly decreased left ventricular end diastolic pressure (LVEDP) compared to arsenic-only treated rats [122].

Naringin (40 and 80mg/kg) fully prevented edema, vacuolization, cytoplasmic eosinophilia, and inflammation, and partially reversed nuclear pyknosis, fibrosis, and collagen deposition in the myocardium of arsenic treated rats [122]. The higher concentrations of naringin significantly decreased serum levels of LDH, CK-MB, AST, ALT, ALP, total cholesterol, triglycerides, LDL and V-LDL, whereas serum HDL was significantly increased compared to arsenic-exposed rats [122]. Treatment with naringin at 40 and 80mg/kg significantly

increased SOD level, GSH level, and Na^+/K^+-ATPase activity, and significantly decreased MDA and NO content compared to the arsenic treated group [122].

Naringin co-treatment (40 and 80mg/kg) significantly increased the activity at mitochondrial complexes I-IV and reduced the appearance of swollen and degenerated mitochondria with dilated perinuclear membrane and vacuolization [122]. Co-treatment with naringin (40 and 80mg/kg) also restored arsenic-induced alterations in cardiac Nrf2 and HO-1, Smad-3 and TGF-β mRNA expression. Naringin (40 and 80mg/kg) also decreased apoptosis and decreased ROS and oxidants (H_2O_2, OH^-, and $ONOO^-$) compared to arsenic treated rats [122].

- Phloretin

Phloretin is a polyphenolic flavonoid found in apples and other sources. Vineetha et al. (2015) exposed H9c2 cells to 5μM arsenic along with phloretin (2.5 and 5μM). Both phloretin concentrations increased cell viability and decreased LDH release compared to arsenic exposed cells. Phloretin (2.5 and 5μM) also restored arsenic- mediated increases in ROS and oxidants (mitochondrial superoxide and combined H_2O_2, OH^-, and $ONOO^-$) and arsenic- mediated reductions in GPx activity, GSH content, SOD activity, and TR activity. Co-

treatment with phloretin at both concentrations decreased Nrf2 to levels near control cells [58].

Phloretin at both concentrations significantly restored arsenic-induced increases in XO and caspase-3 activity, and significantly restored arsenic-induced decrease in aconitase activity. Arsenic exposure significantly elevated calcium content and significantly reduced Ca^{2+}-ATPase activity compared to control cells, whereas treatment with phloretin at both concentrations significantly restored these parameters [58].

Phloretin co-treatment significantly decreased mitochondrial superoxide levels compared to arsenic exposed cells, and phloretin restored activity in mitochondrial complexes I, III, and IV. In this study, arsenic did not result in significant changes in mitochondrial complex II. Phloretin restored arsenic-mediated decreases in ATP content, disruptions in transmembrane potential, and PTP pore opening, as well as arsenic-mediated reductions in oxygen consumption rate. Arsenic exposure also mediated mitochondrial swelling, which was restored with phloretin, and phloretin returned the percentage of necrotic and late apoptotic cells to near control levels [58].

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- Resveratrol

Resveratrol is a polyphenolic stilbene found in red wine. Zhang et al. (2013) studied the effects of resveratrol (8 mg/kg) against arsenic (3mg/kg) in Wistar rats exposed every other day over an 8-day period. Arsenic exposure led to increased arsenic content in the hearts of Wistar rats, whereas resveratrol significantly reduced this accumulation. Compared to arsenic treated rats, rats co-treated with resveratrol demonstrated a significant decrease in markers associated with myocardial injury including LDH, CK, CK- MB, and AST measured in plasma. In addition, resveratrol significantly reversed arsenic-mediated increases in the levels of ROS species and markers of oxidation (H_2O_2, OH-, ONOO-, 8-OHdG, and GSH/GSSG ratio). Resveratrol significantly reduced Ca^{2+} content compared to the arsenic treated group [89]. Resveratrol co-treatment partially restored arsenic-mediated myofibril loss, cardiomyocyte necrosis, and decreased cAMP levels in the heart [89]. Arsenic also mediated down regulation in Nrf2 and HO-1 mRNA gene expression in the heart, both of which were restored with resveratrol. In this study, there was no significant change in NO concentration in arsenic-treated or resveratrol-treated groups, possibly due to the short study duration [89].

Another study conducted by Zhao et al. (2008) tested resveratrol (3mg/kg/) against arsenic (1mg/kg) exposure in BALB/c mice. Animals were exposed to arsenic intravenously on alternate days over a 6-day period. Mice co-treated with resveratrol received resveratrol injections 1-hour prior to arsenic exposure.

In vivo, resveratrol reduced QT elongation, decreased LDH activity in plasma, and increased catalase, GPx, and SOD activities compared to arsenic treated mice. Structural abnormalities following arsenic exposure (cytoplasmic vacuolization, myofibrillar loss, and cardiomyocyte necrosis) were partially prevented with resveratrol co-treatment [124]. Additionally, TUNEL-positive cells were detected with a greater frequency in the hearts of arsenic treated mice compared to control mice and this parameter was dramatically decreased by resveratrol treatment [124].

In vitro, H9c2 cardiomyocytes were pre-treated with 0.1, 1 or 10μM Resveratrol, followed 1-hour later by 10μM arsenic for 24-hours [124]. Resveratrol dose-dependently increased cell viability and dose-dependently reduced LDH release to the medium. Resveratrol dose-dependently reduced apoptosis and DNA damage, and pre-treatment with 10μM resveratrol returned ROS and oxidant levels (H_2O_2, OH-, and ONOO-) to near control levels.

Additionally, pre-treatment with 10μM resveratrol significantly restored arsenic-induced increases in Ca^{2+} accumulation and Caspase-3 activity [124].

- Resveratrol & genistein

Fan et al. (2014) investigated the effects of the flavonoid genistein (50μM) and the stilbene resveratrol (5μM) on primary neonatal rat left ventricular myocytes (NRLVMs) exposed to arsenic (5μM). Cardiomyocytes exposed to 5μM arsenic for 12-hours along with resveratrol or genistein demonstrated neutralized ROS and oxidants (H_2O_2, OH-, and ONOO-) generation and increased mitochondrial transmembrane potential. Resveratrol co-treatment reversed decreases in GSH while resveratrol and genistein co-treatment reversed the reduction in SOD activities induced with 24-hour arsenic exposure. Additionally, genistein and resveratrol enhanced autophagy in 24-hour arsenic treated cardiomyocytes. LC3, a marker of autophagy, was also measured due to the functional relationship between the mediators regulating oxidative stress and autophagy [154]. LC3 was elevated in resveratrol and genistein-arsenic groups compared to the arsenic treated group [117]. Therefore, an enhancement of autophagy in antioxidant-treated groups may represent a beneficial, compensatory response against oxidative stress by removing damaged

organelles (mitochondria), whereas arsenic-treated groups demonstrated lower levels of autophagy and higher levels of apoptosis [117].

Moreover, resveratrol and genistein protected against apoptosis, DNA fragmentation, and decreased cell viability induced by a 24-hour exposure to arsenic. When examined individually, a much lower dose of Resveratrol (5μM) was needed to achieve these effects in cardiomyocytes compared to genistein (50μM).

Although not the focus of the present review, Fan et al. (2014) also investigated the effects of arsenic and resveratrol/genistein in NB4 cancer cells. Arsenic causes cancer cell apoptosis by binding to protein kinase M2 (PKM2), located on the surface of PML/RARA. PKM2 promotes aerobic glycolysis (the "Warburg effect") leading to tumorigenisis and cancer cell proliferation. Arsenic reduces PKM2 activity, thus inhibiting cancer cell growth [64]. It is worth noting that resveratrol/genistein increased the anti-cancer effects of arsenic in NB4 cancer cells by increasing ROS, enhancing mitochondrial transmembrane potential alteration, reducing GSH content and SOD activity, promoting apoptosis, and increasing autophagy beyond the levels achieved by arsenic alone [117].

- *Silybum marianum*

Silybum marianum (SB) is a polyphenolic flavonoid antioxidant of silymarin isolated from the seeds of milk thistle. SB has been used as a hepato-protective agent against arsenic induced liver toxicity [155] and has membrane stabilizing, anti-inflammatory, antioxidant, metal chelation, and cardioprotective qualities [156].

Wistar rats were exposed to 5μM arsenic, 75μM SB, or both for 4-weeks [155]. SB significantly decreased the activities of cardiac enzymes (CK-MB, LDH, AST, ALP), increased heart mitochondrial enzymes (ICDH, SDH, MDH, α-KDH, and NADH hydrogenase), decreased levels of plasma and cardiac lipids (cholesterol, triglycerides, and free fatty acids) and increased phospholipids compared to arsenic exposed rats [155]. Pre-treatment with SB significantly restored arsenic-induced increases in LDL cholesterol and V-LDL cholesterol, and restored arsenic-induced decrease in the level of HDL cholesterol in plasma. Markers of oxidative stress in the heart were significantly reduced by SB-co treatment, whereas GSH content, SOD activity, catalase activity, GPx activity, GST activity, GR activity, and G6PD activity were significantly increased compared to the arsenic treated group. SB partially reversed arsenic-induced changes in mitochondrial morphology and significantly reversed arsenic-induced decreases

in the membrane bound ATPases Na^{+}/K^{+}-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase. SB co-treatment decreased the NOX2 and NOX4 protein levels. NOX family proteins mediate ROS, including H_2O_2 and superoxide, thus decreased NOX expression should decrease anti-oxidant responses. Indeed, SB co-treatment normalized arsenic-altered protein expression of Nrf2, HO-1, and Keap-1 in arsenic treated liver [155].

- Sorbus phnuashanesis (Hante) Hedl

Sorbus phnuashanesis (SPF) is a traditional Chinese herb with high flavonoid antioxidant activity. Yu et al. (2017) exposed BALB/c mice to 5, 10 or 20mg/kg SPF by intraperitoneal injection followed one-hour later with 1mg/kg arsenic by intravenous tail injection for 14-days.

Arsenic caused structural abnormalities in heart tissue including cytoplasmic vacuolization, myofibrillar loss, and cardiomyocyte necrosis compared to control animals, whereas SPF pretreatment significantly alleviated these alterations [114]. ECG analysis demonstrated that SPF prevented arsenic-induced alterations in ST-T wave change and QT-interval prolongation. All SFP concentrations significantly reduced serum cardiac enzymes (CK, CK-MB, and LDH) compared to the arsenic treated group [114].

In vitro, H9c2 cells were pre-treated with 4μM arsenic or co-treated with arsenic and 20μg/mL SPF for 24-hours. SPF significantly maintained cell viability and reduced the release of LDH observed in cells exposed to arsenic for 24-hours. Arsenic reduced the activities of SOD, catalase, and GPx *in vivo* and *in vitro* following 24-hour exposure, whereas SPF significantly alleviated these oxidative stress responses [114]. *In vitro*, SPF significantly decreased arsenic mediated elevation in levels of H_2O_2, OH^- and $ONOO^-$. Pre-treatment with SPF prevented arsenic-induced apoptosis *in vitro* and arsenic-mediated increases in the expression of caspase-3, caspase-8, and caspase-9 both *in vivo* and *in vitro* [114]. SPF also restored arsenic-induced reductions in Bcl-2/BAX protein ratios.

Akt plays a role in arsenic-induced apoptosis and can activate Nrf2. Arsenic significantly reduced the ratio of Akt phosphorylation to total Akt, but this was reversed by SPF co-treatment in H9c2 cells [114]. In agreement, Nrf2 expression was significantly reduced by arsenic, and significantly restored by SPF co-treatment. The Nrf2/antioxidant responsive element (ARE) signaling pathway can induce phase II detoxification enzymes including HO-1. Treatment by SPF was effective at significantly restoring arsenic induced downregulation of HO-1 [114].

- Trichosanthes dioca

Trichosanthes dioca (*T. dioica*) is a dioecious climber found in northern and northeastern India [120]. All parts of the plant have been used for medicinal purposes. The roots contain flavonoids, alkaloids, reducing sugars, saponins, and steroids [120], and the fruit contains flavonoids, glucosides, and alkaloids [119]. Bhattacharya et al. (2014) evaluated the hydroalcoholic extract of *T. dioica* root (TDA) against arsenic toxicity *in vivo*, and Bhattacharya et al. (2013) evaluated the aqueous extract of *T. dioica* fruit (AQTD) against arsenic toxicity *in vivo*.

Although these experiments are reported in separate journals, the experimental design was identical. In both sets of experiments, Wistar rats were orally administered water or the respective antioxidant (TDA at 5 or 10mg/kg [120], or AQTD at 50 and 100mg/kg [119]) every other day for 20 days. On day 21, sodium arsenite was orally administered at 10mg/kg for 8 consecutive days. Arsenic significantly reduced heart and body weight whereas pretreatment with TDA or AQTD dose-dependently ameliorated these effects [120]. White blood cell (WBC) count significantly increased and δ-aminolevulinic acid dehydratase (ALAD), red blood cell (RBC) count, and hemoglobin significantly decreased in arsenic exposed rats, whereas TDA or AQTD significantly and dose-dependently restored these parameters toward control levels [120].

Pre-treatment with TDA or AQTD significantly and dose-dependently decreased CPK, LDH, and total serum cholesterol, and increased HDL cholesterol compared to arsenic treated rats [120]. TDA and AQTD pre-treatment reduced TBARS, increased GSH content and decreased GSSG content compared to arsenic treatment of myocardial tissues [120]. GST, GPx, GR, SOD, and catalase activities were significantly decreased in arsenic-treated rats and significantly modulated by TDA or AQTD in a dose related manner [120]. DNA fragmentation was prevented by TDA or AQTD treatment prior to arsenic exposure [120].

While both the fruit and roots of *T. dioca* were effective against arsenic-induced toxicity, it is worth noting that a significantly lower dose of TDA (5 or 10mg/kg body weight) compared to AQTD (50 or 100mg/kg body weight) was used to achieve similar results.

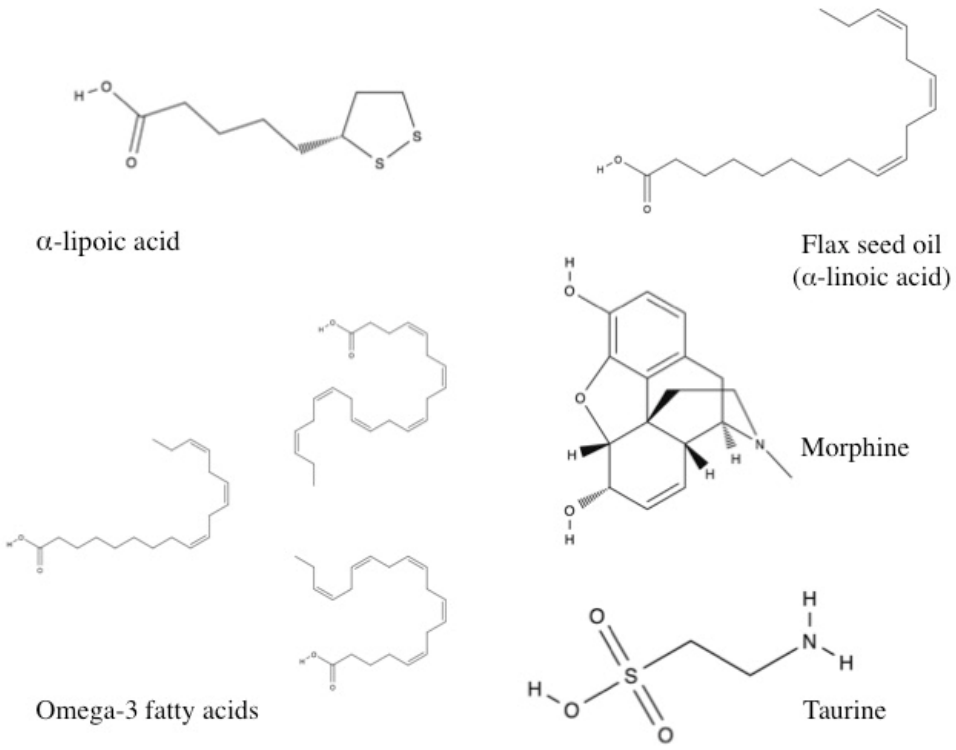


Figure 5. Structure of non-polyphenol antioxidants

iv.ii.ii. Other antioxidants (**Figure 5)**

- α-lipoic acid

α-lipoic acid (LA) is an organosulfur compound derived from octanoic acid. LA contains two sulfur atoms connected by a disulfide bond and has antioxidant properties. Kumazaki et al. (2011) injected Wistar rats with 5mg/kg/day arsenic for 8 weeks in the presence or absence of 35mg/kg/day LA administered orally. Two of the 4 arsenic-exposed rats died suddenly at days 25 and 28 with no earlier symptoms, while all of the LA co-treated rats survived

through the study [111]. Unexpectedly, LA did not prevent arsenic-induced decreases in body weight, decreased AST, or increased urinary excretion of 8-OHDG-an indicator of oxidative stress. This led Kumazaki et al. (2011) to hypothesize that LA prevented death without reducing oxidative stress, possibly through preventing sudden cardiac death [111]. To test this hypothesis, the authors exposed Wistar rats to 0.15, 1.5 and 5mg/kg arsenic for 2-hours followed by 70mg/kg LA in order to assess the EKG profile [111]. Arsenic didn't significantly alter QTc, but the highest arsenic dose caused transient ST-T wave change from 5-30 minutes post infusion, and prolonged the PQ interval [111]. LA prevented alterations in ST-T wave and PQ interval [111].

In a subsequent study, Kumazaki et al. (2013) further explored the cardioprotective effects and chelation potential of LA against QT interval prolongation in a guinea pig model [110]. Hartley guinea pigs were exposed to 1.5mg/kg arsenic intravenously in the presence or absence of LA (0.35, 3.5, or 35mg/kg). The experiment was repeated with LA post-treatment occurring one-hour after arsenic treatment. Both sets of experiments exposed guinea pigs for a 2-hour time frame and ECG readings were taken in live guinea pigs [110].

Continuous infusion with arsenic prolonged QTc interval as early as 60 minutes after dosing, and this effect was dose-dependently and significantly

attenuated by LA co-treatment [110]. Post-treatment with LA also rapidly ameliorated QTc interval prolongation observed with arsenic exposure [110]. The authors suggest that arsenic may induce QT prolongation via the human ether-*a-go-go*-related gene (hERG) channel, thus, Kumazaki et al. (2013) looked at acute exposure on the slowly activating delayed rectifier K^+ current (I_{Ks}) in ventricular myocytes isolated for patch clamping.

Cardiomyocyte exposure to 1μM arsenic inhibited I_{Ks} currents and reduced amperage, both of which were rapidly restored to basal levels with 10μM LA. After washout of LA, arsenic-induced I_{Ks} inhibition returned [110]. Decreased I_{Ks} current prolongs ventricular repolarization, and is one of the most important mechanisms involved in Torsades de Pointes [157].

LA and its reduced form dihydrolipoic acid (DHLA) bind metal ions, thus the chelating potential of LA was investigated with electrospray ionization time-of-flight (ESI-TOF) mass spectrometry analysis. When arbitrary concentrations of LA and arsenic trioxide were mixed, a peak at m/z 450.98 was detected indicating that one molecule of LA (MW 206.3) bound one molecule of As_2O_3 (MW 197.8) with solvent molecules (formic acid (MW 46.0) and protons (MW 1.0)). This peak was not present when the two molecules were analyzed separately [110].

- Flax seed oil

Flax seed oil (FSO) is a rich plant source of polyunsaturated fatty acid alpha-linolenic acid (ALA), a precursor to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which has anticancer, anti-inflammatory, and anti-atherogenic effects [158]. Wistar rats were exposed to 4mg/kg/day arsenic in the presence or absence of 500mg/kg FSO for 45-days [105]. FSO decreased arsenic deposition in the heart and restored structural changes in cardiac tissue compared to arsenic-treated rats [105]. Importantly, FSO significantly reduced CK-MB levels, LDH levels, and TBARS, and significantly increased GSH level and the activities of SOD, GPx, GST, and Catalase compared to arsenic treated rats [105].

- Morphine

Morphine, an opioid, has antioxidant effects including ROS scavenger capacity, NADPH oxidase activity, and increased glutathione levels [159]. Pre-treatment with 1μM morphine protected H9c2 cardiomyocytes against 24-hour 0.5, 1, and 2μM arsenic-induced decreases in cell viability, decreased activity at mitochondrial complex II, and increases in mitochondrial superoxide, and ROS and oxidants (H_2O_2, OH-, and ONOO-) [112]. Arsenic dose-dependently induced

DNA damage, while morphine pre-treatment significantly prevented this deleterious effect [112]. Morphine significantly decreased caspase-3 activity induced with 24-hour exposure to 1 and 2μM arsenic [112]. Forty-eight hour exposure to 2μM arsenic significantly increased the expression of pro-apoptotic BAX and PUMA genes, and significantly decreased the expression levels of anti-apoptotic BCL-2, Survivin, CIAP1, CIAP2, XIAP, and BCL-XL, whereas morphine significantly reversed alterations in BAX, PUMA, BCL2, and Survivin [112]. Additionally, morphine pre-treatment significantly activated NF-κB compared to 0.5-2μM arsenic treated cells [112].

Using morphine in clinical settings is controversial, particularly for chronic or non-cancer pain, based on concerns about addiction, safety, and efficacy [160]. Amini-Kohei et al. (2016) is the only report we are aware of using morphine to prevent arsenic induced cardiovascular toxicity, but it is worth noting that the antioxidant properties of morphine have been reported to protect against methyl-mercury intoxication in rat glioma cells [161] and reverse oxidative damage in neuroblastomas [162], astrocytes [163], and microglial cells [159]. Proposed mechanisms for the antioxidant properties of morphine include direct scavenger activity [159] recovery of GSH levels [162], and/or inhibition of NADPH oxidase activity [164]. Additionally, a recent study suggests that

morphine induces cardioprotection by preventing oxidative stress through mitochondrial Src tyrosine kinase at mitochondrial complex 1 [165].

- Omega-3 fatty acid

Omega-3 fatty acids, found in abundance in fish oil, are long chain polyunsaturated fatty acids with a common chain length of 18, 20, or 22 carbon atoms containing a C=C bond at the third carbon from the end, a carboxylic acid (COOH) at one end and a methyl (CH_3) at the other end [109]. Omega-3s influence oxidative stress by inhibiting the production of inflammatory proteins, decreasing NF-κB activation, and reducing MAPKs [166]. Consuming omega-3 fatty acids, particularly eicosapentaenoic (EPA) and docosahexaenoic acid (DEA), decrease the risk of heart failure, and influence cardiac mitochondrial function by impacting membrane phospholipids [167]. Varghese et al. (2017) investigated the effect of omega-3 fatty acid (50mg/kg) *in vivo* in Wistar rats exposed to 4mg/kg arsenic for 45 days.

Omega-3 co-treatment decreased arsenic deposition in heart tissues and decreased CK-MB [109]. Omega-3s also decreased lipid peroxidation, and increased GPx activity, GSH content, GST activity, SOD activity, and catalase activity compared to arsenic-treated groups [109]. Omega-3 co-treatment

reduced the observed frequency of micronuclei and the presence of abnormal cardiac structures such as swelling near the epicardium, endocardium, and interstitial edema [109]. DHA (100μM) protected against 24-hour, 10μM arsenic-induced LDH release, lipid peroxidation, intracellular Ca^{2+} accumulation, and alterations in MMP in cardiomyocytes [109].

- Selenium

Selenium is an essential trace element present in legumes at concentrations that vary based on soil selenium levels. Selenium and arsenic interact metabolically, resulting in biliary excretion as seleno-bis-*S* (glutathionyl)-arsenium ion ([GS)2AsSe]-) [123]. Krohn et al. (2016) exposed APO E-/- mice to selenium deficient (0.009mgk/kg), selenium adequate (0.16mg/kg), or selenium fortified (0.3mg/kg) diets for 2 weeks, followed by 3 weeks of concurrent exposure to 200ppb sodium arsenite in drinking water [123]. Arsenic exposure significantly increased atherosclerotic plaque formation in the aortic arch of selenium deficient and selenium adequate mice, whereas arsenic exposed mice in the selenium-fortified group demonstrated reduced plaque formation that resembled selenium-fortified controls [123]. Arsenic exposure in conjunction with selenium deficiency led to increased macrophage content in plaques,

indicating decreased plaque stability [123]. Arsenic also increased oxidative stress, measured as decreased concentration of hepatic GSSG, whereas selenium-containing diets significantly restored hepatic GSSG [123]. Arsenic exposure in conjunction with a selenium-deficient diet decreased HDL to LDL ratio compared to the two other diets.

Krohn et al. (2016) report no significant effects of arsenic on total serum cholesterol, LDL cholesterol, triglyceride level, or TBARS. However, the authors report a significant effect of diet on numerous endpoints regardless of arsenic exposure. For example, selenium deficiency increased lesion area in control and arsenic exposed mice. Furthermore, total cholesterol, LDL, and triglycerides were increased in selenium deficient groups regardless of arsenic exposure. This study highlights the negative effects of selenium deficiency alone on cardiovascular health, which resulted in increased plaque formation, as well as the exasperation of plaque formation in the arsenic exposed and selenium deficient condition [123].

- Taurine

Taurine is a sulfonic acid derivative of the amino acid cysteine, which is neither a classic scavenger of ROS nor a regulator of antioxidant defenses [121].

Rather, taurine is believed to serve as a regulator of mitochondrial protein synthesis, which enhances the electron transport chain and protects mitochondria against excessive superoxide generation [156,168]. Taurine is involved in ion channels, transporters, and enzymes, modulates intracellular calcium [169], and has antioxidant properties [170].

Zhao et al. (2008) exposed Wistar rats to 2 mg/kg arsenic for 5 weeks, or 50mg/kg taurine for 2 weeks followed by 2mg/kg arsenic for 5 weeks. The *in vitro* component of this study involved exposing neonatal cardiomyocytes to 25mM taurine, 5μM arsenic, or 25mM taurine followed by 5μM arsenic (after 1-hour) for 24-hours. Overall, taurine increased SOD, catalase, GST, GR, and GPx activities, and increased GSH level and GSH/GSSG ratio compared to the arsenic treated group [121]. Taurine significantly decreased total cholesterol, MDA, and LDH compared to the arsenic treated group, and increased the level of HDL cholesterol to near control levels [121]. Taurine also improved cell viability and decreased apoptosis compared to arsenic-treated rats [121].

Taurine decreased NF-κB pathway phosphorylation [121]. Confirming the involvement of the NF-κB pathway in apoptosis, pre-incubation with the IKK inhibitor PS-1145 prevented arsenic-induced phosphorylation, caspase-3 activation, PARP cleavage, and cardiomyocyte apoptosis [121]. Oxidative stress

can activate MAP Kinases as well as NF-κB, as MAPKs are mediators of cell death due to apoptosis [121]. The protein content of the MAPKs p38 and p-JNK, and to a lesser extent PERK, were elevated in heart tissue and cardiomyocytes following arsenic exposure, and taurine significantly decreased these parameters [121]. Pre-treatment with p38 and p-JNK inhibitors prevented arsenic induced NF-κB activation, suggesting that MAPK activity contributes to NF-κB activation [121].

Arsenic upregulated pro-apoptotic proteins BAX and Bad, and downregulated anti-apoptotic proteins Bcl_2 and Bcl-xL, whereas taurine mitigated these effects [121]. Taurine increased mitochondrial membrane potential, reduced cytosolic cytochrome c content, and decreased intracellular Ca^{2+} compared to arsenic exposed groups [121].

v. Discussion

Arsenic impairs all aspects of mitochondrial function and mitigates apoptosis by elevating ROS. The antioxidants presented in this review largely prevented arsenic-induced pathology *in vivo* and *in vitro*. Compared to arsenic treatment alone, co-treatment with phytonutrient antioxidants restored cardiac function, reduced ROS levels, restored antioxidant activities, reduced apoptosis,

reduced calcium overload, restored ATP content, and restored the activity of mitochondrial complexes. While particularly toxic to mitochondria, arsenic exposure also led to structural abnormalities in the endoplasmic reticulum, lysosomes, and contractile proteins [125]. It is worth noting that antioxidants were reported to be partially effective at restoring arsenic-induced alterations in the morphology of mitochondria and other organelles [122].

v.i. Identifying inconsistencies

Several inconsistencies are worth mentioning. In one study arsenic caused a short-term increase in GSH [118], yet arsenic decreased GSH in all other instances [122]. Since GSH was also reduced at 12 and 24-hours [118], the short term increase was likely a compensatory, beneficial mechanism in response to a specific arsenic dose and time.

Similarly, one study reported increased GPx activity after exposing Wistar rats to 5mg/kg arsenic for 10 days [106]. This contrasts with the majority of studies in which arsenic decreased GPx activity [120]. It is possible that increased GPx activity reported by Hemmati et al. (2017) resulted from the relatively short exposure time (10 days) compared a much longer average exposure time of 25.4 days in other in *vivo* studies that investigated this endpoint.

Additionally, one *in vitro* study reported arsenic-induced increase in Nrf2 level in conjunction with increased HO-1 and increased NQO1 [113]. In contrast, the majority of studies used an *in vivo* model and reported decreased Nrf2 level and decreased level of HO-1 [122]. It is possible that a difference in the *in vitro* cell culture environment caused this variability in the Nrf2 pathway [113]. Interestingly, past research has demonstrated that arsenic-induced effects often fail to follow a typical dose-response pattern [171,172]. This supports the hypothesis that the increase in antioxidant-related pathways is not an inconsistency due to technical variations but a true bimodal response that is dependent on dose and time of exposure to arsenic that can lead to increased upregulation of compensatory pathways. In agreement, past studies of various cell types have reported arsenic-induced upregulation of the Nrf2 pathway [128-132].

In addition to testing antioxidants against arsenic, all of the studies included in this review reported on the impact of antioxidants in the absence of arsenic (untreated conditions) to examine any potential beneficial or detrimental effects on cells/animals exposed to antioxidants alone. As expected, no significant positive or negative effects of antioxidants were reported, with the notable exception of imperatorin and sec-O-glucosylhamaudol, which upregulated the

Nrf2 pathway. This is likely a compensatory response to stimulate additional antioxidant responses, as the Nrf2 pathway contains electrophile DNA elements that can lead to upregulation in the presence of oxidative stress [173].

v.ii. Study comparisons

Based on differences in cell type, experimental animal model, arsenic dose, duration, and experimental design, a direct comparison of antioxidants across studies is not possible in the present review. Only one study compared the effects of different antioxidants (resveratrol vs. genistein) *in vitro* and determined that resveratrol requires a lower dose for the same efficacy against 5μM arsenic [117]. We can make a similar comparison between TDA and AQTD, since the same experimental setup was used by Bhattacharya et al. (2013) and Bhattacharya et al. (2014), revealing that a 10x lower concentration of TDA was effective against 10 mg/kg arsenic. Additionally, studies conducted by Adil et al. (2016) and Muthumani et al (2014) used the same arsenic-dosing paradigm and numerous overlapping endpoints. Thus, we can conclude that 40mg/kg naringin was as effective as 75μM silybum marianum at reversing the effects of 5mg/kg sodium arsenite for the endpoints of total cholesterol, LDL, LDH, AST, ALT, CK-MB, Nrf2, HO-1, SOD activity, GSH activity, NA^+/K^+ ATPase, and altered

mitochondrial morphology [122]. In order to improve our understanding of the capacity of antioxidants to prevent arsenic induced dysfunction, an *in vivo* investigation should be conducted on the pharmacokinetics of arsenic and antioxidants in the heart in relation to cardiotoxicity.

In vitro, there was some overlap in experimental cell type, arsenic concentration, and endpoint, and we can categorize *in vitro* studies conducted on cardiomyocytes by low, moderate, and high arsenic exposure, defined as <2μM, 2 to 5μM and ≥5-10μM. We suggest that a more extensive comparative study be conducted on H9c2 cardiomyocytes testing polyphenols at their optimized concentrations against an intermediate dose of 5μM arsenic.

v.iii. Limitations

Despite the overall strength of the studies included in this review, we acknowledge a level of variability in the strength of study design and data reporting of included studies (**Table S1**). All included studies were peer reviewed, had clearly stated experimental objectives, and all *in vitro* studies identified the source and/or isolation method of cells. Among *in vivo* studies, 92% fully disclosed the characteristics of the experimental model including animal species, source, gender, and physical characteristics. In addition, 68.4% of studies

analyzed in this review specified that animals were randomly assigned to experimental groups, and all studies used commonly accepted experimental methods.

However, no studies specifically conducted a power analysis to justify the size of experimental groups or the number of technical replicates in experiments. Additionally, no studies reported effect sizes. In the absence of effect sizes, the relevance of antioxidant effects cannot be comparatively assessed.

With regard to data assessment and reporting, 88% fully reported the statistical methods used, and 91% of these studies used appropriate methods.

The average number of citations per article was 12.1 ± (20.5), and the average H index (a measure of productivity and citation impact) for journals in which articles were published was 86.8± (50.8).

While several aspects of these studies are lacking, particularly in the area of complete data reporting, our results of the studies analyzed provide compelling evidence that antioxidants protect against arsenic-induced mitochondrial dysfunction, and make a strong case for performing additional studies that directly compare the efficacy of different antioxidants.

A potential limitation for the applicability of this research in a real world context is low bioavailability of polyphenol antioxidants. Polyphenols are

defined and categorized based on the nature of their chemical skeletons. Major classes are phenolic acids, flavonoids, stilbenes, and lignins. Flavonoids are the most abundant polyphenol in our diets, and classes of flavonoids are based on the degree of oxidation of the oxygen heterocycle [145,174]. Flavonoid classes are isoflavones, flavonols, flavones, flavanols, proanthocyanidins, and anthocyanins [145].

Assessing polyphenol bioavailability is not straightforward. Measuring polyphenol antioxidant quantities is a useful way to compare food items, yet polyphenol content cannot necessarily be extrapolated to bioavailability or health claims [175]. For example, consuming flavonoid-rich food will only slightly increase the flavonoid content of plasma due to the process of polyphenolic metabolism, in which flavonoids are broken down into smaller phenolic acids, absorbed and metabolized to form glucuronides and sulfide conjugates [175]. Conjugated forms of polyphenols appear in circulation and can have altered bioactivity from the parent compound [175].

Individual variability in levels of the enzymes involved in polyphenol metabolism also impact overall bioavailability of polyphenols [175]. Some polyphenols are absorbed in the stomach or small intestine, while others are absorbed in the colon following metabolism by gut microflora. Variations in gut

microbe populations further alter polyphenol bioavailability at the individual level [175].

Despite challenges predicting polyphenol bioavailability, studies have reported on concentrations of polyphenols circulating in plasma, or the amount of polyphenol measured in urine, even though these observations do not necessarily correlate with the amount of a compound that reaches a target site to exert a desired effect [175]. Although an indirect method to assess bioavailability, a more accurate measure of polyphenol bioavailability is to assay for markers of peroxidative damage *in vivo* following polyphenol consumption. However, a limited number of studies on polyphenol bioavailability have been conducted using this method in humans. The available data suggests that among flavonoids, isoflavones have the highest bioavailability, with absorption between 33-100%, followed by flavonols (12-41%), and flavanones (11-16%) [174]. Tea catechins and anthocyanins are among the polyphenols with the lowest absorbability, while catechins have intermediate absorption characteristics (lower than isoflavones and higher than flavonols [175].)

An additional potential limitation on applying these findings in arsenic-affected regions relates to the ability to extrapolate *in vitro* and animal model experimental results to human populations. The dose ranges of both arsenic and

ameliorative antioxidant treatments employed in the reviewed studies vary significantly. Although the doses of arsenicals used in these studies may be considered high, they are applicable in the context of human exposures. Humans have been acutely and chronically exposed to similar concentrations of arsenicals environmentally [176] and in the clinical treatment of acute promyelocytic leukemia [177]. Furthermore many epidemiological studies have demonstrated the pathological effects of long-term accumulation of arsenicals in human bodies at high doses [43,50].

We also acknowledge a lack of consensus in the research on the benefits of antioxidant supplementation in humans. Observational studies suggest that consuming greater amounts of antioxidant-rich foods decreases the risks of cardiovascular diseases, stroke, and cancer, but it is not entirely clear if these effects are due to antioxidants themselves, due to other substances in fruit and vegetables, or a result of other behaviors (such as exercise) among individuals who consume a higher volume of antioxidant-rich foods.

While laboratory studies consistently demonstrate that antioxidants stabilize free radicals, many long-term clinical trials of antioxidant supplementation have failed to conclusively demonstrate a benefit of antioxidant supplementation. It is worth noting, however, that several recent double blind, placebo-controlled

studies support the role of antioxidant amelioration of oxidative stress in humans. For example, 12-week supplementation with 100mg resveratrol significantly ameliorated arterial stiffness and decreased serum diacron-reactive oxygen metabolites (d-ROMS)-a measure of oxidative stress- in type 2 diabetics [107], supplementation with 500mg/day resveratrol significantly decreased oxidative stress (measured as serum MDA) in subjects with ulcerative colitis [108], and supplementation with α-lipoic acid in type-2 diabetics significantly increased SOD and GPx, and decreased MDA in subjects compared to baseline measures and compared to the placebo-control group [109]. Thus, recent clinical trials provide evidence that antioxidant's ability to ameliorate oxidative stress translates to human subjects. Additionally, several clinical trials have reported mild benefits of selenium pill supplementation on measures of arsenic toxicity [178-180], and Krohn et al. (2016) are currently planning a clinical trial supplementing the diet of arsenic-exposed individuals in Bangladesh with selenium fortified lentils [181]. Also if interest, poor nutrient status and malnutrition among arsenic exposed individuals has been associated with increased rates of cardiovascular diseases [182], skin lesions [183], and Blackfoot disease [184]. This further supports the importance of consuming an adequate diet with sufficient calories and nutrients.

v.iv. Future directions

Given the limitation of low polyphenol bioavailability, research has been conducted on combining polyphenols to improve bioavailability. Evidence suggests that combined polyphenols can have a synergistic effect, particularly with regard to anti-cancer properties. For example, combining EGCG and curcumin allowed for an 8-fold dose reduction in EGCG in the mixture for the same therapeutic effect [172]. Additionally, Fan et al. (2014) reported that the combination of resveratrol and genistein was more effective against NB4 cancer cells than either polyphenol alone [117]. Therefore, based on these aforementioned observations in cancer, it is conceivable that combination therapies involving more than one polyphenol can exert synergistic effects in reversing cardiovascular pathology following arsenic exposure.

Another promising area of research is on improved polyphenol extraction and preservation techniques. Polyphenols are found in many natural sources complexed with sugars or proteins, or in polymerized derivatives [172]. To achieve effective isolation, proper selection of a solvent for extraction is necessary to maintain molecular integrity, and recovery depends on time and temperature (high temperature, long extraction times, and alkaline environment cause degradation) [172].

More recently, encapsulation and nanoformulation have been proposed as potential solutions, as these techniques can improve both stability and bioavailability of polyphenols [172]. Nanoformulations include nanosuspensions, solid lipid nanoparticles, liposomes, gold nanoparticles, micelles, and polymeric nanoparticles. Nanoencapsulaton can increase intracellular concentration and allow slow and sustained polyphenol release [185].

Nanoformulations have been developed for several polyphenols for use against cancer cells in pre-clinical trials. Ellagic acid encapsulated nanoparticles were developed for use in oral cancer with improved results [186]. In addition, EGCG nanoformulations have been used against human prostate cancer cells, human melanoma cells, mouse models of melanoma, breast cancer cell lines, and breast cancer cells isolated from patients [187]. Silibinin nanoparticles have been used against human hepatocellular cells and carcinoma cell lines [188], and resveratrol nanoformulations have been used against human prostate cancer cell lines [189], murine melanoma cells [190], rat glioma cells [191], human head and neck cancer cells [192], and human ovarian cells [193]. Importantly, resveratrol has also been developed into a mitochondrial targeting drug to improve anti-cancer ability [194]. Sassi et al. (2014) linked resveratrol derivatives with an o-linked mitochondria targeting 4-triphenlyphoshponlumbutyl group to

selectively kill fast growing cells [194]. Additionally, a mitochondrial targeted prototype nanoformulation of genistein was developed by Pham et al. (2013) and is reported to increase cytotoxicity through the intrinsic apoptotic pathway against hepatic and colon carcinoma [195].

Although not focused specifically on arsenic-mediated cardiovascular dysfunction, several recent clinical trials have assessed the benefit of polyphenols on general cardiovascular effects. For example, Richter et al. (2017) reported that isoflavone-containing soya protein supplementation significantly reduced brachial diastolic blood pressure compared to a lower dose of soya protein (but not compared to control) [196]. Even more compelling, Tenore et al. (2017) demonstrated that supplementation with microencapsulated annurca apple polyphenol extracts, trade-marked as 'AppleMetS' significantly decreased LDL cholesterol, significantly increased HDL cholesterol and significantly decreased total cholesterol compared to control subjects in a randomized parallel group placebo-controlled 2 week study [185].

The present review identifies 21 compounds with antioxidant properties that are effective against arsenic-mediated mito-toxic effects in cardiovascular tissue. We believe that the current limitations in bioavailability of polyphenol antioxidants are surmountable. Also of interest, mitoquinone (MitoQ) is a

mitochondrially targeted molecule designed as an antioxidant to block mitochondrial oxidative damage [197]. MitoQ selectively accumulates in mitochondria and is currently being pursued as a therapy for degenerative conditions such as Parkinson disease [198]. While MitoQ has not been tested against arsenic-induced cardiotoxiciy, it has demonstrated cardioprotective properties against Doxorubicin (DOX), which is used for treating various cancers despite severe cardiovascular side effects including congestive heart failure, arrhythmias, and cardiomyopathy [199]. Importantly, mitochondria-targeting nanoformulations represent a compelling avenue of research for preventing arsenic-induced cardiovascular toxicity in a clinical context and among individuals chronically exposed to arsenic through ground water or APL therapy.

As a preliminary step, we suggest a pre-clinical approach of comparing antioxidant abilities of several combinations of polyphenol antioxidants to prevent arsenic-induced mito-toxic effects in H9c3 cardiomyocytes, while simultaneously increasing toxicity to NB4 cells. We suggest a focus on the mitochondrial endpoints of the Nrf2 pathway and associated antioxidants, caspase activation, apoptosis and associated Bcl-2/BAX expression, GSH activity, mitochondrial and overall ROS, calcium overload, Na^+/K-ATPase activity,

activity at mitochondrial complexes, OCR, and ATP generation. We believe the aforementioned proposed studies will give a comprehensive view of antioxidant capacity to prevent mitochondrial dysfunction. Following antioxidant selection, we recommend developing a mitochondrial-targeted nanoformulation for use in H9c3 and NB4 cell lines and Wistar rat models. As mitochondrial targeting nanoformulations of resveratrol and genistein have already been developed, adapting these formulations for use against arsenic-induced cardiotoxicity may be the most time- and resource-effective approach to developing real world solutions.

v.v. Conclusions

Antioxidants have the potential to improve the cardiovascular health of millions of people chronically exposed to elevated arsenic concentrations through contaminated water supplies or used as lifesaving cancer treatments. While consuming foods containing antioxidants is a healthful and useful practice for the prevention of cardiovascular disease, additional research is needed on proper compounding, encapsulation and nanoformulations that prevent antioxidant degradation and improve polyphenol pharmacokinetics in order to counteract arsenic-induced cardiovascular toxicity in a real-world context.

Table S1
Study Quality

Criterion		Yes (n)	No (n)	Yes %	Average (SD)
1.	Was the study purpose clearly stated?	25	0	100%	-
2.	Number of technical replicates (*in vitro*)	-	-	-	5.8 (1.6)
3.	Number of endpoints assayed	-	-	-	14.6 (9.6)
4.	Were source and type of cells/animals listed?	23	2	92%	
5.	What is the number of experimental groups?	-	-	-	5.8 (2.2)
6.	Did study specify the number of animals per group? (*in vivo*)	24	1	96%	6.7 (1.6)
7.	Was gender of animals specified? (*in vivo*)	25	0	100%	-
8.	Were animals randomly assigned to experimental groups?	13	6	68.4%	-
9.	Was a power analysis performed?	0	24	0%	-
10.	Was variability assessed:				
	Between subjects?	25	0	100%	-
	Within subjects?	0	24	0%	-
11.	Was statistical method fully reported?	22	3	88%	-
12.	Was statistical method appropriate?	20	2	91.3%	-
13.	Was numerical data reported?	18	7	72%	-
14.	Were effect sizes reported?	0	25	0%	-
15.	What statistics were reported for primary outcomes?				
	Mean(SE)	11	14	44%	-
	Mean(SEM)	14	11	56%	-
16.	Was funding source reported?	17	8	68%	-
17.	Average number of citations/article	-	-	-	12.1 (20.5)
18.	H Index	-	-	-	86.8 (50.8)

Chapter 3.
Arsenic methylation capacity and metabolic syndrome in U.S. NHANES 2013-2014

i. Abstract

Arsenic methylation capacity is associated with metabolic syndrome and its components among highly exposed populations. However, this association has not been investigated in low to moderately exposed populations. We investigated arsenic methylation capacity, measured as urinary proportion of arsenic species and methylation indices, in relation to the clinical diagnosis of metabolic syndrome in a low arsenic exposure population. Additionally, we compared arsenic methylation patterns present in our sample to methylation patterns among more highly exposed populations.

Using linear regression models adjusted for relevant biological and lifestyle covariates, we report a positive association between increased arsenic methylation and metabolic syndrome among women with normal body mass index (BMI). Methylation patterns in our sample were similar to reports of methylation capacity among highly exposed populations. To our knowledge this is the first investigation of methylation capacity with respect to metabolic syndrome in a low exposure population. Additionally, we report that gender and BMI significantly modify the effect of arsenic methylation on metabolic

syndrome. Future studies should evaluate the effectiveness of current arsenic standards on subclinical biomarkers of cardiovascular disease in a model that establishes causality.

ii. Introduction

Exposure to arsenic has been epidemiologically linked to myriad health conditions including cardiovascular disease, diabetes, and cancer [200]. Exposure to inorganic arsenic occurs through the consumption of contaminated food and water sources, and arsenic biotransformations in the liver increase the presence of highly toxic methylated arsenic metabolites [27,57,139]. Methylated arsenicals increase (ROS) to a greater extent than inorganic arsenic, thus, arsenic methylation may be associated with increased disease prevalence [27].

Although the World Health Organization (WHO) recommends a maximum contaminant level (MCL) for arsenic of 10 ppb, global arsenic concentrations in untreated waters can reach several hundred to several thousand ppb, contributing to as much as 21.4% of all causes of mortality in highly exposed regions of the world [34].

Chronic exposure to arsenic has been associated with elevated risk of cardiovascular disease (CVD) endpoints including ischemic heart disease

incidence and mortality in several American Indian populations with water sources measured up to 21 ppb arsenic [9] and among populations in Bangladesh, over half of which consume ground water that substantially exceeds 50 ppb arsenic [34]. Increased rates of cerebrovascular disease prevalence have been observed among residents of Taiwan with water sources that can exceed 300 ppb arsenic [201], and increased severity of atherosclerosis was observed among subjects in rural Taiwan with median arsenic concentrations in drinking water between 700 and 930 ppb [177]. Additionally, a recent meta-analysis demonstrated a positive association between arsenic exposure and hypertension among 11 cross-sectional studies of subjects with various levels of arsenic exposure [67].

Arsenic exposure is also associated with a diagnosis of metabolic syndrome, a risk factor for CVD. Metabolic syndrome is defined as the presence of at least 3 out of 5 components including elevated fasting glucose, hypertension, elevated triglycerides, large waist circumference, and low HDL cholesterol [202]. Inorganic arsenic exposure was associated with a clinical diagnosis of metabolic syndrome, elevated plasma glucose, and elevated blood lipids among residents in an industrial region of Taiwan exposed to arsenic both occupationally and through water sources measured up to 16 ppb [49]. The

association between arsenic and metabolic syndrome has not been investigated in a population exposed to low arsenic concentrations.

In addition to arsenic exposure, arsenic methylation capacity is a recognized risk factor for arsenic toxicity, and may contribute to CVD susceptibility. Inorganic arsenic (iAs) undergoes biotransformation through its monomethylated form (MMA) and dimethylated form (DMA), both of which are more toxic than the parent compound [27]. Arsenic methylation capacity is measured as the proportion of methylated arsenicals in urine or as a ratio of MMA:iAs (termed primary methylation index or PMI) and ratio of DMA:MMA (termed secondary methylation index or SMI). Arsenic methylation facilitates urinary excretion, yet also increases the bioavailability of toxic methylated arsenicals [27]. Interestingly, methylation capacity is not highly associated with inorganic arsenic exposure [203], but is influenced, by genetics, gender, age, and BMI [204,205].

Incomplete arsenic methylation is characterized by a pattern of higher urine proportion MMA, lower urine proportion DMA, increased PMI, and decreased SMI, whereas more complete arsenic methylation is characterized by a pattern of lower urine proportion MMA, higher urine proportion DMA, decreased PMI and increased SMI. Although only a limited number of studies

have investigated the impact of arsenic methylation on cardiovascular disease or metabolic syndrome, the available evidence suggests that complete methylation may be a contributing factor [53]. Importantly, this association has not been investigated in a relatively low arsenic-exposed population. Indeed, reports of adverse health outcomes associated with arsenic exposure and arsenic methylation have originated from regions where arsenic concentrations are unregulated or significantly under-regulated and thus exceed current safety guidelines. In contrast, we assessed the relationship between arsenic methylation and pre-clinical biomarkers of cardiovascular disease in a general sample of the U. S. where despite widespread compliance with current treatment standards for arsenic in food and water, appreciable concentrations of arsenic are still detectable in the blood and urine of U.S. residents [54,206-208].

The present study uses U.S. NHANES 2013-2014 data to investigate the association between urine %MMA, %DMA, and indices of primary methylation (PMI) and secondary methylation (SMI), with respect to a clinical diagnosis of metabolic syndrome.

iii. Materials and Methods

iii.i Study population

Data for this study were acquired from the U.S. National Health and Nutrition Examination Survey (NHANES) website [209]. Complete protocols for sample selection are described elsewhere [210]. In brief, NHANES is a national, population-based, cross-sectional study representing the civilian, non-institutionalized U.S. population. Demographic information is collected via survey and a physical examination is performed on all subjects. Biological samples are collected from nationally representative subsamples. The present study draws from subjects selected for the environmental subsample containing urinary arsenic concentrations which comprises approximately one third of the total U.S. NHANES population, and subjects selected to provide blood samples in the morning fasting session, comprising approximately half of the total U.S. NHANES population.

iii.ii. Exclusion Factors

Pregnancy was an exclusion factor in the present study based on the influence of pregnancy on several measures used to assess metabolic syndrome (e.g. waist circumference, blood pressure, and cholesterol measures). Pregnancy status was determined by NHANES personnel using the Icon 25 hCG test kit (Beckman Coulter) administered to women age 20-44. In the present study, we

excluded subjects under the age of 20 because arsenic metabolism is believed to differ between youth/adolescents and adults [211] and metabolic syndrome is rare among subjects under 20. We also excluded subjects who were missing data on metabolic syndrome components and covariates (age, gender, race/ethnicity, smoking status, poverty income ratio (PIR), urine creatinine, urine arsenobetaine, or BMI). Our final analytical sample contained 957 subjects.

iii.iii. Speciated arsenicals

Concentrations of iAs(III), iAs(V), (MMA(III+V)), and (DMA(III+V)) were determined by NHANES personnel using high performance liquid chromatography (HPLC) to separate the species coupled to Multi-Element Inductively Coupled Plasma-Mass Spectrometry (ICP-DRC-MS) [212]. The lower limits of detection (LLOD) were 0.12μg/L (iAs(III)), 0.79μg/L (iAs(V)), 0.20μg/L (MMA(III+V)) and 1.91μg/L (DMA(III+V)). For our analysis, subjects with arsenic species below detection were assigned a concentration of LLOD/ √2, representing 29.8%, 24.5%, and 26.9%, for iAs(III), DMA, and MMA, respectively. We eliminated iAs(V) from subsequent analysis as 98.3% of samples were below detection limits for this measure. Total arsenic concentration, % arsenic species,

and indices of methylation were calculated for the present study using the following formulas:

$$\text{Total Arsenic} = \text{iAs+MMA+DMA}$$

$$\%\ \text{MMA} = ((\text{MMA/total arsenic})*100)$$

$$\%\text{DMA} = ((\text{DMA/total arsenic})*100)$$

$$\text{PMI} = \text{MMA/iAs}$$

$$\text{SMI} = \text{DMA/MMA}$$

PMI and SMI were right skewed and log adjusted for all subsequent analyses. Urinary % MMA and DMA were right skewed but non-log normal, thus untransformed values were used in subsequent analyses. Non-log adjusted values are presented for descriptive purposes.

iii.iv. Metabolic syndrome components

Physical and biological data pertaining to metabolic syndrome were obtained from the NHANES website [213]. In the present study, a diagnosis of metabolic syndrome was given to subjects with at least 3 of the following conditions; fasting glucose $\geq$100 or medication to control glucose; systolic or diastolic blood pressure $\geq$ 130/85mmHg or medication taken to control blood pressure; triglycerides $\geq$150mg/dL; waist circumference $\geq$ 102 cm (males) or $\geq$ 88

cm (females); and HDL-cholesterol < 40mg/dL (males) or <50mg/dL (females) [202].

iii.v. Fasting glucose

Glucose concentrations were determined by NHANES personnel using the Beckman Unicel DxC800 Synchron method. The complete protocol is published elsewhere [212]. In brief, oxygen electron circuits determine the rate of oxygen consumption, which is directly proportional to the concentration of glucose in the sample. In the present study, subjects were classified as having elevated fasting glucose if their fasting serum glucose reached or exceeded 100mg/dL, if they reported currently taking insulin, or if they reported currently taking a diabetic pill.

iii.vi. Hypertension

After resting in a seated position for 5 minutes, 3 or 4 consecutive blood pressure measurements were taken by NHANES personnel. In the present study we eliminated the first measurement unless it was the only measurement available, and averaged the remaining blood pressure measurements in accordance with recommendations from the American Heart Association council on High Blood Pressure Research [214]. Subjects whose average blood pressure

reached or exceeded 130/85 mmHg were considered to have elevated blood pressure. Subjects currently taking blood pressure lowering medication were also classified in the high blood pressure group, as determined by questionnaire administered by NHANES personnel.

iii.vii. Elevated triglycerides

Triglycerides were measured by NHANES personnel in refrigerated serum using the Beckman Unicel DxC800 Synchron timed-endpoint method [212].

iii.viii. Waist circumference.

Waist circumference measurement was determined by NHANES trained health technicians and was measured at the high point of the iliac crest at minimal respiration to the nearest 0.1 cm [215].

iii.ix. Low HDL Cholesterol

The Roche Cobas 6000 chemistry analyzer method was used by NHANES personnel to measure serum cholesterol [212]. In brief, this endpoint reaction

measures HDL-cholesterol using an ion specific electrode (ISE) and photometric measuring system.

iii.x. Covariates

Age, , gender, race/ethnicity and smoking status were determined by questionnaire during household interviews administered by trained and NHANES certified personnel [213]. In the present study, smokers were defined as subjects who responded in the affirmative to smoking cigarettes 'every day' or on 'some days'. A measure of creatinine concentration was determined by NHANES personnel for each urine sample via the enzymatic method using the Roche Cobas 6000 Analyzer [212], and was considered as an independent variable [216]. Arsenobetaine, a measure of non-toxic arsenic from seafood, was measured by NHANES personnel via HPLC -ICP-DRC-MS [212]. Poverty income ratio (PIR) was calculated by NHANES personnel according to the Department of Health and Human Service (HHS) guidelines by dividing family income by the poverty guidelines specific to the survey year [212]. BMI measurements were collected by trained NHANES health technicians and are expressed as weight in kilograms divided by height in meters squared rounded to one decimal place. For the present study, BMI was categorized as normal

(BMI<25.0), overweight (25.0≤BMI<30.0), or obese (BMI≥30.0). Complete protocols for all NHANES data collection methods are published elsewhere [212].

iii.xi. Statistical analysis

Descriptive and statistical analyses were conducted with SPSS v32. When combining data from more than one sample, using the sampling weight of either subsample is not recommended by NHANES as it could result in unreliable estimates [217]. Thus, the complex sampling design was ignored because the study population belonged to two subsamples (environmental subsample and fasting subsample).

We used logistic regression to assess the relationships between urine %MMA, urine %DMA, PMI, and SMI with metabolic syndrome. Covariates were evaluated in the literature and statistically using Mann-Whitney-U test, Kruskal Wallace test, χ^2 test, or student-t test, and statistically relevant or biologically justified covariates were included in the final adjusted model. We investigated plausible interactions and stratified our models by BMI and gender. We considered using BMI as a dependent variable but based on statistical collinearity between metabolic syndrome and BMI, we selected metabolic

syndrome as our dependent variable and stratified by BMI category. A sensitivity analysis was conducted among subjects who exceeded baseline measures for arsenic species (MMA n=700, DMA n=732, PMI n=585, SMI n=681), and a subgroup analysis was conducted on non-diabetic subjects (n=871). We also considered the effect of controlling for total arsenic and/or arsenobetaine, and the inclusion of urinary creatinine as an independent variable in our main models. Urinary creatinine was considered as an independent variable rather than a covariate because adjusting for creatinine has been suggested to confound the relationship between total urinary arsenic and arsenic metabolism [216].

iv. Results

Table 1 provides study participant characteristics. The mean age of the study population was 47 years. Approximately 51% of subjects were male and 46% of subjects were white. Three hundred and twenty six subjects (34.1%) met the clinical diagnostic criteria for elevated fasting glucose, 425 subjects (44.4%) had elevated blood pressure or were taking medication to lower blood pressure, 261 subjects (27.3%) had elevated triglycerides, 526 subjects (55.0%) had large waist circumference, and 288 subjects (30.1%) had low HDL cholesterol. Overall, 331 (34.6%) of subjects met the inclusion criteria for metabolic syndrome. Our

sample had 9.68% (±6.70), 11.69% (±9.94), and 78.63% (±9.94) urine iAs, MMA, and DMA, respectively **(Table 1).** The average total urinary arsenic in our sample of the U.S. NHANES 2013-2014 population was 15.67 μg/L.

Table 1
Study participant characteristics

	Mean (SD) or N (%)	
Age (years)	47.44	(15.55)
Sex		
Male	491	(51.30%)
Race/Ethnicity		
Non-Hispanic White	442	(46.2%)
Mexican American	120	(12.5%)
Non-Hispanic Black	204	(21.3%)
Non-Hispanic Asian	87	(9.1%)
Other Race	104	(10.8%)
PIR[a]	2.2	(1.61)
PIR < 1	278	(29.0%)
Smoker	196	(20.5%)
BMI[b] (kg/m^2)	29.01	(7.34)
Normal	313	(32.7%)
Overweight	290	(30.3%)
Obese	354	(36.9%)
Metabolic syndrome components		
Fasting glucose (mg/dL)	102.21	(34.94)
Systolic blood pressure (mmHg)	122.09	(17.53)
Diastolic blood pressure (mmHg)	68.99	(11.55)
Serum triglycerides (mg/dL)	139.93	(235.32)
Waist circumference (cm)	99.12	(16.91)
HDL cholesterol (mg/dL)	52.63	(15.28)
Urinary Arsenic Species		
iAs III[c] (μg/L)	0.51	(0.45)
MMA III + V[d] (μg/L)	0.63	(0.55)
DMA III+ V[e] (μg/L)	4.68	(5.38)
% iAs[f]	9.68	(6.70)
% MMA[g]	11.69	(5.95)
% DMA[h]	78.63	(9.94)

PMI[i]	1.89	(1.89)
SMI[j]	10.44	(20.72)
Number of Metabolic syndrome components		
0		179 (18.7%)
1		233 (24.3%)
2		214 (22.4%)
3		193 (20.2%)
4		104 (10.9%)
5		34 (3.6%)
Less than 2		626 (65.4%)
3 or more		**331 (34.6%)**

[a] Poverty Income Ratio was calculated according to the Department of Health and Human Service (HHS) guidelines by dividing family income by the poverty guidelines specific to the survey year [212], [b] Body Mass Index was calculated as weight in kilograms divided by height in meters squared rounded to one decimal place, [c] trivalent inorganic arsenic, [d] trivalent and pentavalent monomethylarsonous acid, [e] trivalent and pentavalent dimethylarsonous acid, [f] (iAs / (iAs+MMA+DMA)*100), [g] (MMA / (iAs+MMA+DMA)*100), [h] (DMA/ (iAs+MMA+DMA)*100), [i] MMA/iAs, [j] DMA/MMA.

iv.i. Covariate association with arsenic measures and metabolic syndrome

We were interested in the relationship between biologically relevant, literature-justified covariates and our independent and dependent variables. We first examined the association between continuous and categorical covariates with measures of arsenic methylation (**Table 2**). Gender was significantly associated with all measures of arsenic methylation ($p<0.05$). Women had significantly lower %MMA, significantly higher %DMA, and significantly increased PMI and SMI compared to men. Race/ethnicity, BMI, smoking status and PIR were associated with at least one arsenic measure (**Table 2).** White subjects had significantly higher %MMA compared to Black and Asian subjects,

White subjects had significantly lower %DMA compared to Black subjects, and White subjects had significantly higher PMI compared to Mexican-American and Black subjects.

Smokers had a significantly higher PMI compared to non-smokers, and subjects with a PIR < 1 had higher SMI compared to subjects with PIR ≥ 1. Subjects with obese BMI demonstrated a pattern of increased arsenic methylation: significantly lower %MMA and significantly higher %DMA compared to subjects with normal BMI. Age was significantly associated with all measures of arsenic methylation and urine arsenobetaine was significantly associated with %MMA and SMI (**Table 2**). These data suggest that when considered independently, female gender, non-White race, poverty, obesity, increased age, increased seafood consumption (measured as arsenobetaine), and dehydration (measured as urine creatinine) were associated with increased arsenic methylation.

Table 2
Distribution of arsenic measures by covariate

	Arsenic Measures Mean (SD)			
	%MMA[a]	%DMA[b]	PMI[c]	SMI[d]
	***p*-Value[e]**	***p*-Value**	***p*-Value**	***p*-Value**
Total (n=957)	11.69(5.95)	78.63(9.94)	1.89(1.89)	10.44(20.72)
Gender	**<0.001**	**<0.001**	**0.003**	**<0.001**
Male (n=491)	12.41(6.02)	76.85(10.13)	1.73(1.67)	10.13(27.32)
Female (n=466)	10.93(5.79)	80.51(9.41)	2.05(2.08)	10.76(9.81)

Race/ Ethnicity[f]	**<0.001**	0.098	**<0.001**	**0.001**
White (n=442)	12.53(6.07)	78.11(10.41)	2.18(2.05)	9.00(9.40)
Mex.Amer[g].(n=120)	11.30(6.02)	78.20(10.28)	1.38(1.50)[h]	9.71(7.13)
Black (n=204)	10.61(5.52)[h]	79.22(9.24)	1.64(1.83[h]	13.24(40.59)
Asian (n=84)	10.17(5.64)[h]	81.69(8.02)[h]	1.64(1.42)	13.58(16.07)
Other (n=107)	11.96(5.96)	77.60(9.98)	1.71(1.89)	9.19(7.23)
Smoker	0.141	0.962	**0.009**	0.268
No (n=761)	11.54(5.90)	78.61(10.01)	1.84(1.87)	10.78(22.95)
Yes (n=196)	12.27(6.14)	78.677(9.72)	2.09(1.95)	9.08(7.13)
PIR[i]	0.053	0.194	0.988	**0.049**
<1 (n=278)	12.24(5.97)	77.86(10.16)	1.90(1.92)	10.89(35.18)
≥1 (n=679)	11.46(5.93)	78.94(9.85)	1.88(1.88)	10.25(9.98)
BMI	**<0.001**	**<0.001**	0.780	**<0.001**
Normal[j]	12.72(6.44)	76.67(10.81)	1.85(1.76)	9.29(11.56**)**
Overweight[k]	12.00(5.51)	78.16(9.50)	1.89(1.73)	8.98(7.31)
Obese[l]	10.52(5.67)	80.75(9.09)	1.91(2.11)	12.66(31.52)

	[B(Se) Beta ***p*-Value**[m]]			
Age	-0.04(0.01)-0.116 **.002**	0.15(0.02) 0.250 **.001**	0.00(0.00) 0.182 **.001**	0.00(0.00) 0.159 **.001**
Creatinine[n]	1.59(0.59) 0.078 **.011**	-2.74(0.97) -0.018 **.002**	-0.04(0.04)-0.037 .220	-0.07(0.03)-0.072 **.008**
Arsenobetaine[o]	-2.60(0.29)-0.252 **.001**	4.61(0.51) 0.267 **.001**	0.00(0.02) 0.000 .995	0.15(0.02) 0.292 **.001**

[a] urine % of monomethylarsonous acid, [b]urine % of dimethylarsonous acid, [c] primary methylation index, [d] secondary methylation index, [e] *p*-values obtained from Mann-Whitney U Test comparing two groups or Kruskal-Wallis Test comparing more than two groups (α=0.05), [f] post hoc analysis conducted with Tukey's test, [g] Mexican American, [h] Significant difference compared to White race, (Tukey's post hoc test), [i] Poverty Income Ratio – calculated by dividing family income by the poverty guidelines specific to the survey year [212], [j] BMI<25.0, [k] 25.0≤BMI<30.0, [l] BMI≥30.0,[m] *p*-values obtained from bootstrapped linear regression (α=0.05), [n] creatinine is used to normalize for hydration, [o] arsenobetaine is a non-toxic form of arsenic found in seafood

Next we assessed the association between covariates and our dependent variable (metabolic syndrome). Clinical diagnostic cutoff levels differ for men and women, therefore, we stratified our sample by gender. Age, BMI, smoking status, and PIR were significantly associated with metabolic syndrome in both

men and women, whereas urinary creatinine and race/ethnicity were only significantly associated with metabolic syndrome in women **(Table 3).**

Table 3
Association between metabolic syndrome and covariates

	Metabolic syndrome status			**Metabolic syndrome status**		
	(No) N=334	**(Yes)** N=157	***p*-Value**[a]	**(No)** N=292	**(Yes)** N=174	***p*-Value**
	Men Mean (SD) or N			**Women** Mean (SD) or N		
BMI			**<0.001**			**<0.001**
Normal[b]	148	17		132	16	
Overweight[c]	132	55		67	36	
Obese[d]	54	85		93	122	
Smoker			**<0.001**			**<0.001**
Yes	87	42		41	26	
Non	247	115		251	148	
PIR[e]			**<0.001**			**<0.001**
<1	38	96		85	59	
≥1	238	119		207	148	
Race/Ethnicity			0.472			**0.049**
White	150	72		133	87	
Mex.-Amer.[f]	38	22		41	19	
Black	73	38		50	43	
Asian	30	13		28	13	
Other	43	12		40	12	
Age	44.4(16.7)	53.6(15.0)	**<0.001**	44.2(16.3)	53.3(15.0)	**<0.001**
Creatinine[g]	133.1(76.7)	134.1(73.4)	0.767	98.0(69.2)	109.4(70.1)	**0.036**
Arsenobetaine[h]	7.9(19.4)	6.7(19.9)	0.266	10.4(37.2)	6.6(18.8)	0.477

Notes: [a] *p*-Values obtained from 2-way χ^2 test or one-way ANOVA for categorical variables and Man Whitney U test for continuous covariates, [b] BMI<25.0, [c] 25.0 ≤ BMI < 30.0, [d] BMI ≥ 30.0, [e] Poverty Income Ratio – calculated by dividing family income by the poverty guidelines specific to the survey year [212], [f] Mexican American, [g] creatinine is used to normalize for hydration, [h] arsenobetaine is a non-toxic form of arsenic present in seafood

iv.iii. Logistic regression

Our crude analysis suggested that in men, decreased urine %MMA, increased urine %DMA, and increased SMI were significantly associated with increased odds of metabolic syndrome whereas in women, increased %DMA and increased SMI were significantly associated with increased odds of metabolic syndrome (data not shown).

Next we adjusted our gender stratified models for statistically relevant and literature justified covariates [22]. We adjusted gender-stratified models for age (continuous), race (White vs. non-White), poverty status (PIR < 1 vs. PIR ≥ 1), and smoking status (dichotomous) (**Table 4**). In the gender stratified, adjusted model, decreased %MMA was significantly associated with increased odds of metabolic syndrome in men, whereas increased SMI was significantly associated with increased odds of metabolic syndrome in women.

Table 4
Binary logistic regression, fully adjusted[a]

		Men			Women	
IV	R^2	OR [95% CI]	***p*-value[b]**	R^2	OR [95% CI]	***p*-value**
%MMA[c]	0.079	0.960 [0.927, 0.994]	**0.021**	0.085	0.974 [0.940, 1.010]	0.153
%DMA[d]	0.073	1.015 [0.995, 1.037]	0.150	0.087	1.020 [0.998, 1.044]	0.078
PMI[e]	0.073	0.656 [0.358, 1.203]	0.173	0.081	0.956 [0.543, 1.683]	0.876
SMI[f]	0.073	1.636 [0.834, 3.209]	0.152	0.090	2.148 [1.048, 4.402]	**0.037**

[a] adjusted for age (continuous), poverty income ratio (PIR<1 vs. PIR≥1) – calculated by dividing family income by the poverty guidelines specific to the survey year [212], race (white vs. non-white), and smoking status (dichotomous), R^2 represents Cox & Snell R^2 value, [b] *p*-value obtained from binary logistic regression, [c]

urine % of monomethylarsonous acid, [d] urine % of dimethylarsonous acid, [e] primary methylation index, [f] secondary methylation index

Based on the significant overlap between subjects with metabolic syndrome and overweight/obese BMI [218], we stratified by BMI category rather than adjusting for BMI. (**Table 5**) provides the fully adjusted model among male subjects stratified for BMI category, and (**Table 6**) provides fully adjusted, BMI stratified values for women. The data suggest that BMI confounds the association between arsenic methylation and metabolic syndrome in men: our stratified regression model returned non-significant *p*-values for all associations of arsenic methylation and metabolic syndrome in men. In contrast, the data suggest that BMI modifies the effect of arsenic methylation on metabolic syndrome in women: reduced urine %MMA and increased SMI were significantly associated with metabolic syndrome in women with normal BMI, whereas the opposite trend occurred in women with overweight BMI (significantly increased %MMA and reduced SMI), and there was no significant association between arsenic methylation and metabolic syndrome in obese women. However, it should be noted that the sample size for women with normal BMI and metabolic syndrome was relatively small (n=16 women) and the confidence interval is large.

We next assessed the possibility of biologically plausible interactions in our gender-stratified model (age*smoking, age*PIR, and PIR*smoking) and report no significant interactions (data not shown).

Controlling for total arsenic, including urinary creatinine as an independent variable in logistic regression models [216] and excluding diabetic subjects had no significant impact on our results. In contrast, sensitivity analysis in which we excluded subjects below detection limits for arsenic species eliminated the significant effect of arsenic methylation on metabolic syndrome in subjects with overweight BMI, but did not alter the significance of results among subjects with normal BMI. Additionally, controlling for seafood consumption (measured as urine arsenobetaine), in obese subjects resulted in a significant association between increased SMI and increased odds of metabolic syndrome, whereas the association was not significant in the model prior to adjusting for arsenobetaine. This suggests that the role of arsenic methylation on metabolic syndrome may have been masked by the effect of seafood consumption, whereas controlling for seafood consumption revealed a true association between increased arsenic methylation (SMI) and metabolic syndrome (**Tables S1 and S2**).

Table 5
Binary logistic regression
DV: Metabolic syndrome in men, fully adjusted model[a]

	Normal BMI[b]			Overweight BMI[c]			Obese BMI[d]		
IV	R^2	OR [95% CI]	*p*-value[e]	R^2	OR [95% CI]	*p*-value	R^2	OR [95% CI]	*p*-value
%MMA[f]	0.073	0.974 [0.888, 1.068]	0.577	0.101	1.019 [0.958, 1.083]	0.554	0.112	0.979 [0.913, 1.049]	0.549
%DMA[g]	0.074	1.019 [0.963, 1.078]	0.519	0.102	0.986 [0.951 1.022]	0.429	0.110	1.007 [0.966 1.050]	0.728
PMI[h]	0.072	1.179 [0.230, 6.043]	0.843	0.099	0.926 [0.312, 2.748]	0.890	0.110	0.995 [0.333, 2.979]	0.993
SMI[i]	0.072	1.470 [0.238, 9.081]	0.678	0.106	0.477 [0.129, 1.757]	0.266	0.110	0.929 [0.275, 3.144]	0.906

[a] adjusted for age (continuous), Poverty Income Ratio (PIR<1 vs. PIR≥1) – calculated by dividing family income by the poverty guidelines specific to the survey year [212], race (White vs. non-White), and smoking status (dichotomous), [b] BMI<25.0, [c] 25.0≤BMI<30.0, [d] BMI≥30.0, R^2 represents Cox & Snell R^2 value, [e] *p*-value obtained from binary logistic regression, [f] urine % monomethylarsonous acid, [g] urine % dimethylarsonous acid, [h] primary methylation index, [i] secondary methylation index

Table 6
Binary logistic regression
DV: Metabolic syndrome in women, fully adjusted model[a]

	Normal BMI[b]			Overweight BMI[c]			Obese BMI[d]		
IV	R^2	OR [95% CI]	*p*-value[e]	R^2	OR [95% CI]	*p*-value	R^2	OR [95% CI]	*p*-value
%MMA[f]	0.182	0.826 [0.701, 0.973]	**0.022**	0.219	1.113 [1.016, 1.218]	**0.021**	0.057	0.966 [0.919, 1.015]	0.174
%DMA[g]	0.167	1.085[0.999, 1.178]	0.053	0.203	0.951 [0.903 1.003]	0.063	0.054	1.018 [0.986 1.052]	0.273
PMI[h]	0.160	0.644 [0.365, 1.135]	0.128	0.187	1.149 [0.912, 1.446]	0.239	0.049	0.992 [0.879 1.120]	0.896
SMI[i]	0.175	11.485 [1.371, 96.196]	**0.024**	0.220	0.089 [0.011, 0.704]	**0.022**	0.064	2.590 [0.926, 7.243]	0.070

[a] adjusted for age (continuous), Poverty Income Ratio (PIR<1 vs. PIR≥1) – calculated by dividing family income by the poverty guidelines specific to the survey year [212], race (White vs. non-White), and smoking status (dichotomous), [b] BMI<25.0, [c] 25.0≤BMI<30.0, [d] BMI≥30.0, R^2 represents Cox & Snell R^2 value, [e] *p*-value obtained from binary logistic regression, [f] urine % monomethylarsonous acid, [g] urine % dimethylarsonous acid, [h] primary methylation index, [i] secondary methylation index

v. Discussion

Ours is the first study to investigate the association between arsenic methylation and metabolic syndrome in a population with relatively low arsenic exposure. We report that increased arsenic methylation is associated with increased odds of metabolic syndrome among women with normal BMI.

v.i. Oxidative stress and inflammation

Metabolic syndrome is associated with a clustering of abnormalities that lead to increased cardiovascular risk. Inflammation is an important cellular mechanism that links metabolic syndrome to cardiovascular disease [218]. For example, obesity (a central component of metabolic syndrome) is associated with overexpression of tumor necrosis factor α (TNF-α), a cell signaling protein involved in systematic inflammation [219], and high fat intake contributes to increased oxidative stress and nuclear factor (NF-κB) activation, a proinflammatory signaling pathway [220]. Additionally, the inflammatory marker C-reactive protein (CRP) statistically enhances the relationship between metabolic syndrome and coronary heart disease events [221]. Furthermore, oxidative stress is believed to contribute to metabolic syndrome [222], hyperglycemia [223], dyslipidemia [224], and obesity [225].

Oxidative stress has also been convincingly linked with exposure to arsenic. Indeed, exposure to inorganic arsenic and its metabolites can generate ROS and free radicals, hydroxyl radicals, nitric oxide, and superoxide anion in a variety of cell lines [57,226]. Additionally, a human study conducted among a highly arsenic exposed population in China reported that arsenic-exposed subjects demonstrated higher oxidative stress compared to control subjects (measured as increased serum levels of lipid peroxide) [227]. Importantly the same study reported a significant correlation between oxidative stress and methylated arsenic metabolites [227].

Thus, there is evidence for an association between arsenic and oxidative stress, as well as evidence of a link between oxidative stress and metabolic syndrome. Furthermore, our outcome of a positive association between increased arsenic methylation and metabolic syndrome is consistent with past investigations conducted among populations with higher overall arsenic exposure. Indeed, in regions of Taiwan with exposure to industrial arsenic as well as water sources measured up to 16 ppb, Wang et al. (2007) reported a positive association between total arsenic and metabolic syndrome [49] and Chen et al. (2012) reported that low primary methylation (low % MMA) was associated

with a higher risk for metabolic syndrome in a population exposed to median arsenic concentrations of 700-930ppb [53]. Although a minority of findings suggest that incomplete methylation (high urinary proportion of MMA) is a stronger contributor to cardiovascular disease [51] and hypertension [228], this may result from differences in the valence state of measured arsenicals. For example, studies demonstrating elevated toxicity of complete methylation have included trivalent and pentavalent species in calculations of urinary arsenic proportion, whereas studies reporting the opposite trend measured only the less toxic pentavalent species [51] or failed to specify the valence states of measured arsenicals [228]. In the present study, we report that increased arsenic methylation is associated with increased odds of metabolic syndrome in specific subgroups.

v.ii. Increased toxicity of methylated species

Following ingestion and tissue distribution, a series of biotransformations in the liver reduce arsenate (iAs(V)) to arsenite (iAs(III)) and arsenic methyltransferase enzymes mediate the sequential addition of methyl ions from S-adenosylmethionine, alternated with arsenic species' reductions [18]. Thus,

ingested inorganic arsenic is sequentially converted to its mono- and di-methylated forms and removed through the urinary system (**Figure 1**).

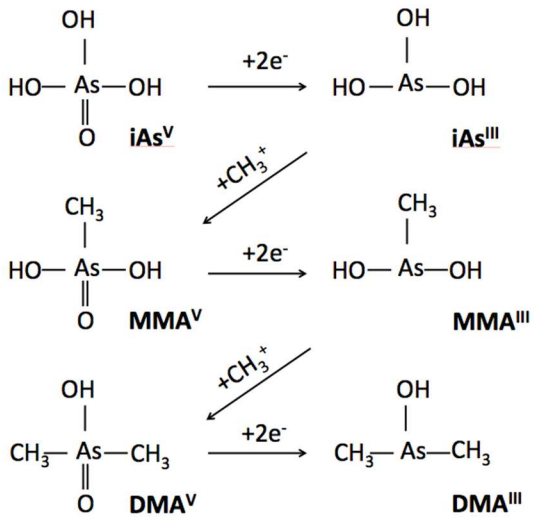


Figure 1. Arsenic biomethylation

Once considered a detoxification reaction, a growing body of evidence demonstrates that arsenic methylation significantly increases toxicity and that trivalent species are more toxic than pentavalent species [25,57]. Individual variability in arsenic biotransformation is measured as the proportion of each arsenic species in urine, or by calculating PMI and SMI. Variation in urine metabolites have been associated with a variety of arsenic-related diseases

[51,229] and may reflect differential bioavailability of arsenicals across individuals [230].

v.iii. Arsenic distribution patterns

Other notable findings from the present study are the similarities in arsenic methylation patterns in our sample of the U.S. compared to regions with higher exposure, as well as similarities with respect to individual variation in methylation capacity [203]. Urinary proportions of arsenic species found in the present study are consistent with reports that most individuals have 10-30% iAs, 10-20% MMA, and 60-80% DMA, regardless of their arsenic exposure level [203]. Our sample had 9.68% (±6.70), 11.69% (±9.94), and 78.63% (±9.94) urinary iAs, MMA, and DMA, respectively. The average total urinary arsenic in the U.S. NHANES 2013-2014 population was 15.67 μg/L, considerably lower than the total urinary arsenic reported in the aforementioned studies of pre and post-clinical CVD outcomes, where total mean urinary arsenic has ranged from 53.6 μg/L among women from southwest U.S. and northwest Mexico [231] to 580 μg/L among a northern Chile population [203]. Relative consistency in average arsenic methylation capacity among different populations irrespective of arsenic exposure was also reported by Hopenhayn-Rich et al. (1996), who detected only

subtle (2-3%) differences in mean urine proportion of MMA and DMA between populations exposed to inorganic arsenic at levels differing by 500ppb [203].

In contrast to the relative consistency of arsenic methylation patterns between study populations, considerable variability has been reported in methylation capacity between individuals in the same population [22]. For example, subjects in San Pedro ranged from approximately 40% to over 90% urinary DMA and from 7% to 40% urinary MMA [203]. In the present study, urine percent MMA ranged from below detection limits to 34.22%, and urine percent DMA ranged from 41.16% to 98.74%.

v.iv. Gender and BMI

Past studies suggest that variability in methylation capacity is due, in part to variations in genetic polymorphisms, as well as age, BMI, and gender [204,205]. Our results also demonstrate an effect of gender and BMI on arsenic metabolism. In the present study, we report that increased arsenic methylation is associated with increased odds of metabolic disorder in gender and BMI stratified samples of the U.S. NHANES population adjusted for relevant covariates.

Our finding that gender modified the effect of arsenic methylation on the odds of metabolic syndrome is supported by previous research conducted by Lindberg et al. (2007), who reported that women in Bangladesh had higher methylation efficiency than men during childbearing ages (20 to 60 years) [204]. Similarly, European women between the ages of 20 and 60 had an increased rate of arsenic methylation [205]. The authors of these studies speculate that sex hormones are partially responsible for increased rates of methylation in women, as it is specific to women of childbearing age [204,205]. This is a plausible explanation for some of the observed differences in metabolic syndrome outcomes based on gender in the present study, as 79% of women in our sample were between the ages of 20-60.

The present study also demonstrated a significant effect of BMI on arsenic methylation. Subjects with obese BMI expressed a pattern of increased arsenic methylation: significantly lower %MMA and significantly higher %DMA compared to subjects with normal BMI. Since increased arsenic methylation is generally associated with increased disease status, we expected to see a significant association between arsenic methylation and metabolic syndrome in obese subjects. Contrary to our expectations, we report a significant association

between increased arsenic methylation and metabolic syndrome in women with normal BMI.

We were interested in which clinical symptoms characterized metabolic syndrome in subjects who had metabolic syndrome and normal BMI. Among these 16 women, we found that 13 had elevated fasting glucose, 11 had elevated triglycerides, 14 had elevated blood pressure, and 12 had low HDL cholesterol. The most common combination of symptoms were elevated blood pressure and elevated glucose in combination with low HDL cholesterol (n=7) or elevated triglycerides (n=6). Despite the small sample size, ours is the first report of BMI as an effect modifier of in the association between arsenic methylation and metabolic syndrome.

v.v. Selection of adjustment factors

It is worth noting that we adjusted for covariates similar to those used in other studies assessing the relationship between arsenic exposure or arsenic methylation and various aspects of metabolic syndrome. These varied somewhat based on the specific research question, but generally included age, gender, BMI, smoking status, alcohol use, diabetes, waist to hip ratio, serum lipid levels, urine creatinine, and urine arsenobetaine [9]. In the present study, we adjusted for age, poverty status, smoking status, and race, and stratified by gender and BMI

category. These covariates are used in comparable studies with biological relevance verified in the literature [232]. Additionally, we report no significant effect of controlling for urine creatinine, total arsenic, or eliminating diabetic subjects. We did, however, determine that limiting our analysis to subjects above detection limits for MMA eliminated the statistical significance between low arsenic methylation and metabolic syndrome in overweight subjects. This suggests that the apparent protective effect of overweight BMI was not a true effect, but rather, was due to a large number of subjects in the overweight BMI group with undetectable arsenic levels. Although we used a commonly accepted practice by assigning a value of the LLOD/ $\sqrt{2}$ to subjects below detection limits, this erroneously resulted in an apparent protective effect of overweight BMI, which we deemed implausible. Sensitivity analysis revealed that this was likely an artifact of our statistical method, and not a true effect.

We also probed our model by controlling for arsenobetaine and found that in women with obese BMI, the role of arsenic methylation on metabolic syndrome was masked by the effect of seafood consumption. By re-evaluating our final model after controlling for seafood consumption (i.e. adjusting for arsenobetaine), we detected a significant association between increased arsenic methylation (SMI) and metabolic syndrome. Navas-Acien et al. (2011) report that

seafood is a significant determinant of urine concentrations of total arsenic and arsenic species. Arsenobetaine is a non-toxic organic form or arsenic present in high levels in seafood that is excreted unchanged via the kidneys. Navas-Acien et al. (2011) also propose eliminating subjects who have consumed seafood in the last 24-hour from statistical analyses as a further safeguard from misclassifying arsenic exposure. In the present study, statistically controlling for arsenobetaine altered the statistical significance of results among female subjects with obese BMI, whereas controlling for arsenobetaine in women with normal BMI did not alter the significant association between decreased urine %MMA and increased SMI with increased odds of metabolic syndrome. While arsenic concentrations from seafood are an important consideration for statistical analysis, the most important source of arsenic exposure in terms of health outcomes is contaminated water sources.

v.vi. Policy implications

An important implication of our study is the relevance of public water testing and treatment. The U.S. follows EPA recommendations for a MCL of 10 ppb inorganic arsenic in public water. The EPA is responsible for regulating contaminants in public drinking water. This authority was established through

the Safe Drinking Water Act (SDWA) of 1974, which requires the EPA to define and set legal limits for contaminants, develop a testing schedule, and determine appropriate testing methods and techniques [233]. Under this act more than 90 contaminants are regulated at specified MCLs [233]. The SDWA requires that an MCL be reliably testable, achievable by the best technology available, and result in health risks deemed acceptable, defined as 1 cancer death per 10,000 persons or less [233]. Based on the findings of the National Academy of Sciences (NAS), the current MCL is estimated to result in 5 deaths per 10,000 persons, failing to meet EPA safety standards. The current MCL is justified based on a 1996 amendment to the SDWA, which allows for a recommended MCL to be revised in light of a benefit-cost analysis [233]. The NAS initially supported an MCL of 3 ppb based on their analysis of relevant toxicology and epidemiology research [234]. Using the NAS recommendations and a cost-benefit assessment, the EPA originally proposed an MCL of 5 ppb inorganic arsenic, which was later revised to 10 ppb inorganic arsenic. Unfortunately the current MCL fails to consider cardiovascular outcomes, diabetes, and preclinical indictors of CVD that are associated with arsenic exposures below 10 ppb, as demonstrated in the literature. The present study suggests that the current MCL may not be sufficiently protective against metabolic syndrome, as we detected a significant

association between arsenic methylation and metabolic syndrome, even when adjusting for total arsenic exposure (**Table S1 and S2**). Further study is needed to assess the adequacy of current water standards for arsenic in the U.S. in terms of preclinical biomarkers of CVD.

In addition to the possibility that current standards fail to protect individuals in the U.S. from arsenic related diseases, not all waters are subject to EPA standards, potentially placing an unequal health burden on regions with naturally higher arsenic concentrations and greater reliance on unregulated water sources. For example, rural areas of the U.S. with a higher proportion of un-piped water report much higher concentrations for groundwater arsenic. A survey of 102 homes using domestic wells in rural areas of Nevada averaged 356 μg/L arsenic pre-treatment and 87 μg/L arsenic following reverse osmosis treatment [8], and arsenic concentrations were measured between 10 and 61 ppb on rural reservation lands in Arizona [9]. Even populations using piped water on Reservation lands are at risk of consuming high concentrations of arsenic. Indeed, health-related violations relating to one or more substance above the MCL, were reported among 46% of public water systems on Reservations, compared to just 7% in public water systems in the remainder of the U.S. [233]. Despite the real and significant risk to rural and Native American populations,

rural areas represent a small fraction of the total U.S. population and are thus unlikely to represent a large proportion of the U.S. NHANES sample used in the present study.

Looking beyond the U.S., global arsenic regulations range from 6 ppb in Sweden to 50 ppb in Bangladesh, though there is vast disparity in regional infrastructure that supports water testing, reporting, and remediation for arsenic. For example, despite regulations, a 2009 survey of 15,000 randomized households in Bangladesh estimated that 22 million people consume water exceeding 50 ppb and 5.6 million people consume water exceeding 200 ppb [235]. However, there is no reliable estimate of the global scope of arsenic exposure due to delayed health effects, poor reporting, and low levels of awareness in some regions [236]. Based on findings from the present study we cannot rule out the possibility that exposure to water meeting the MCL of 10 ppb inorganic arsenic does not contribute to metabolic syndrome.

v.vii. Limitations

Important limitations in the present study include reliance on secondary data and the use of self-reported data for several measures (e.g. diabetes, smoking status, and the use of medication). Additionally, as our sample originated from

two different subsamples, we were unable to use the U.S. NHANES weighting factors and are thus unable to apply our findings to the general population. Finally, our study design allows for an interpretation of correlation but not causation. Despite these limitations, this is the first study to examine urinary arsenic methylation with respect to metabolic disorder in the U.S. We also acknowledge that our stratified sample sizes were small, and these outcomes should be assessed in a larger population.

vi. Conclusion

In summary, in a country where arsenic levels are regulated at 10 ppb in public drinking water, we report a significant association between increased arsenic methylation capacity and metabolic syndrome in women with normal BMI. Our sample represents a relatively low exposure population, and the association between arsenic methylation to metabolic syndrome may be significantly greater in regions of the world that lack the infrastructure for water testing and treatment and in regions of the U.S. that remain highly exposed to arsenic, such as American Indian reservations and rural communities relying on untreated waters from private domestic wells. We emphasize the need for studies that highlight the impact of policy enforcement on urinary arsenic, and health outcomes, as well as additional studies that consider the contribution of

gender, BMI, and other factors that may modify the association of arsenic methylation to pre- and post-clinical cardiovascular disease status in a model that establishes causality.

Table S1
Subgroup and sensitivity analyses in fully adjusted[a] binary logistic regression model of women
IV: %MMA[b]
DV: Metabolic Syndrome

	Normal BMI[c]			Overweight BMI[d]			Obese BMI[e]		
Additional Criteria	**R^2**	**OR [95% CI]**	***p*-value[f]**	**R^2**	**OR [95% CI]**	***p*-value**	**R^2**	**OR [95% CI]**	***p*-value**
Exceeding baseline for MMA[g]	0.177	0.747 [0.588, 0.950]	**0.017**	0.346	1.060 [0.947, 1.186]	0.311	0.057	0.976 [0.918, 1.037]	0.427
Non diabetic subjects	0.182	0.826 [0.701, 0.973]	**0.022**	0.291	1.113 [1.016, 1.218]	**0.021**	0.056	0.976 [0.928, 1.026]	0.340
Adjusted for total arsenic	0.198	0.817 [0.689, 0.968]	**0.020**	0.221	1.111 [1.014, 1.216]	**0.024**	0.057	0.966 [10.920, 1.016]	0.177
Adjusted for arsenobetaine[h]	0.187	0.817 [0.690, 0.970]	**0.021**	0.231	1.102 [1.000, 1.210]	**0.040**	0.075	0.960 [0.913, 1.009]	0.109
Adjusted for creatinine[i]	0.182	0.825 [0.699, 0.973]	**0.002**	0.220	1.112 [1.015, 1.218]	**0.022**	0.067	0.965 [0.917, 1.014]	0.161

Notes: [a] adjusted for age (continuous), gender, race (dichotomous, White vs. non-White) smoking status (dichotomous), and poverty status (PIR<1 vs. PIR≥1), [b] Urine % monomethylarsonous acid, [c] BMI<25.0, [d] 25.0 ≥BMI<30.0, [e] BMI≥30.0, R^2 represents Cox & Snell R^2 value, [f] *p*-value obtained from binary logistic regression, [g] urine MMA ≥0.20µg/L, [h] arsenic in seafood, [i] measure of hydration

Table S2
Subgroup and sensitivity analyses in fully adjusted[a] binary logistic regression model of women
IV: SMI
DV: Metabolic Syndrome

	Normal BMI[b]			Overweight BMI[c]			Obese BMI[d]		
Additional Criteria	**R^2**	**OR [95% CI]**	***p*-value[e]**	**R^2**	**OR [95% CI]**	***p*-value**	**R^2**	**OR [95% CI]**	***p*-value**
Exceeding baseline for MMA[f]	0.220	8.309 [1.065, 64.850]	**0.043**	0.306	0.223 [0.022, 2.797]	0.207	0.050	2.290 [0.707, 7.417]	0.167
Non diabetic subjects	0.175	11.485 [1.371, 96.196]	**0.022**	0.220	0.089 [0.011, 0.074]	**0.022**	0.061	2.124 [0.738, 6.110]	0.162
Adjusted for total arsenic	0.188	12.244 [1.324, 113.201]	**0.027**	0.221	0.093 [0.012, 0.739]	**0.025**	0.064	2.580 [0.092, 7.230]	0.072
Adjusted for arsenobetaine[g]	0.180	12.550 [1.440, 109.76]	**0.022**	0.229	0.119 [0.014, 0.999]	**0.050**	0.083	3.115 [1.080, 8.960]	**0.035**
Adjusted for creatinine[h]	0.175	11.635 [1.370, 98.791]	**0.025**	0.220	0.090 [0.011, 0.715]	**0.023**	0.073	2.663 [0.938, 7.560]	0.167

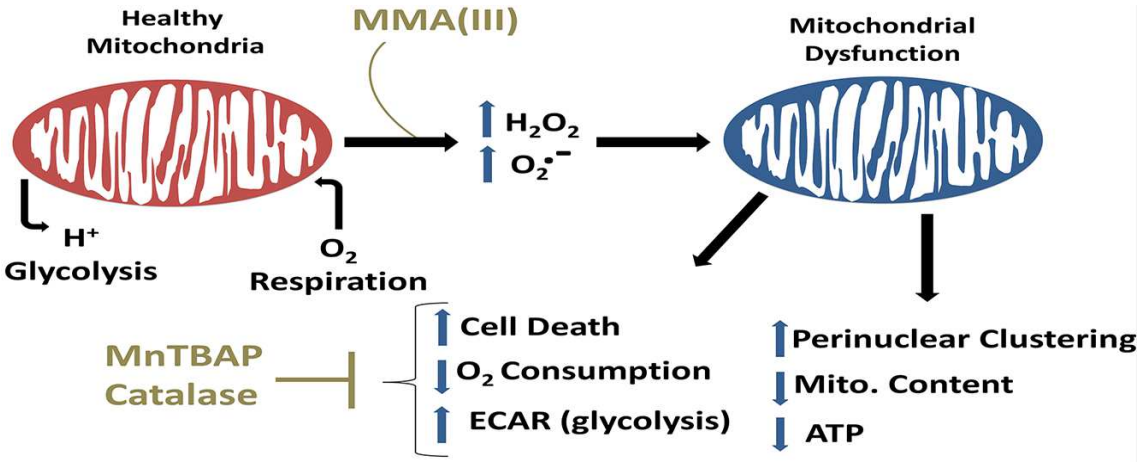
Notes: [a] adjusted for age (continuous), gender, race (dichotomous, White vs. non-White) smoking status (dichotomous), and poverty status (PIR<1 vs. PIR≥1), [b] Secondary methylation index, [c] BMI<25.0, [d] 25.0 ≥BMI<30.0, [e] BMI≥30.0, R^2 represents Cox & Snell R^2 value, [f] *p*-value obtained from binary logistic regression, [g] urine DMA ≥1.91μg/L, urine MMA ≥0.20μg/L, [h] arsenic in seafood, [i] measure of hydration

Chapter 4. Monomethylarsonous acid, but not inorganic arsenic, is a mitochondria-specific toxicant in vascular smooth muscle cells

i. Abstract

Arsenic exposure has been implicated as a risk factor for cardiovascular diseases, metabolic disorders, and cancer, yet the role mitochondrial dysfunction plays in the cellular mechanisms of pathology is not fully elucidated. To investigate arsenic-induced mitochondrial dysfunction in vascular smooth muscle cells (VSMCs), we exposed rat aortic smooth muscle cells (A7r5) to inorganic arsenic (iAs(III)) and its metabolite monomethylarsonous acid (MMA(III)) and compared their effects on mitochondrial function and oxidative stress. Our results indicate that MMA(III) is significantly more toxic to mitochondria than iAs(III). Exposure of VSMCs to MMA(III), but not iAs(III), significantly decreased basal and maximal oxygen consumption rates and concomitantly increased compensatory extracellular acidification rates, a proxy of glycolysis. Treatment with MMA(III) significantly increased hydrogen peroxide and superoxide levels compared to iAs(III). Exposure to MMA(III) resulted in significant decreases in mitochondrial ATP, aberrant perinuclear clustering of mitochondria, and decreased mitochondrial content. Mechanistically, we observed that mitochondrial superoxide and hydrogen

peroxide contribute to mitochondrial toxicity, as treatment of cells with MnTBAP (a mitochondrial superoxide dismutase mimetic) and catalase significantly reduced mitochondrial respiration deficits and cell death induced by both arsenic compounds. Overall, our data demonstrates that MMA(III) is a mitochondria-specific toxicant that elevates mitochondrial and non-mitochondrial sources of ROS.



Graphical abstract

ii. Introduction

Exposure to arsenic has been associated with cancer, diabetes, cardiovascular disease, and metabolic syndrome [200,237-239]. It has been suggested that arsenic alters common pathways such as those involved in oxidative stress and inflammatory signaling, which underlie a variety of arsenic-associated diseases [237,240-242]. The generation of ROS in particular is a major pathological mechanism induced by arsenic and is involved in the pathogenesis

of cancer [243], insulin resistance [244], metabolic syndrome [245], and cardiovascular disease [242].

Arsenic has been shown to induce oxidative stress in numerous cell lines, including human vascular smooth muscle cells (VSMCs) [246-249,250], by causing the release of iron from ferritin [251], or by altering antioxidant activity and increasing inflammation [252]. Additional tissue pathology in the vascular endothelium caused by exposure to arsenic includes an increase in oxidative stress caused by increased NADPH oxidase activity in VSMCs and endothelial cells, leading to an increase in superoxide and hydrogen peroxide levels [246], and a vicious cycle of dysregulated calcium and nitric oxide signaling. Arsenic-induced ROS couples with nitric oxide (NO) producing peroxynitrite [253] and decreases the bioavailability of NO to vascular endothelium and smooth muscle, which likely contributes to cardiovascular complications. ROS have also been shown to result in cell damage and cell death in numerous cell lines and models of arsenic-induced disease pathology [254-256].

Inorganic arsenic (iAs(III)) undergoes biomethylation in the liver to form the toxic intermediate metabolites monomethyl-arsonous acid (MMA(III)) and dimethylarsonous acid (DMA(III)). These trivalent organic arsenic species have demonstrated elevated toxicity relative to iAs(III) in several studies [27,139,257]

and may be responsible for many of arsenic's pathological effects. Indeed, cells with higher rates of arsenic metabolism show increased susceptibility to oxidative damage to DNA and induction of tumorigenesis [27,258]. These organic arsenic species can also promote cytotoxicity and tissue pathology by decreasing the levels of several antioxidants. For instance, in comparison to iAs(III), much lower concentrations of MMA(III) over shorter periods of time can alter the activity of glutathione peroxidase and catalase, induce expression of stress proteins and metallothioneins, and increase ROS levels [139]. Other proposed mechanisms for the enhanced toxicity of MMA(III) include greater rates of cell permeation and accumulation [259], differences in subcellular distribution, and selective toxicity towards subcellular compartments such as the mitochondria [250].

Several studies have investigated the effects of arsenic on VSMCs, which comprise the medial cell layer of vascular arteries and are known to be exposed to arsenic through contact with blood plasma. *In vivo*, it has been reported that iAs(III) increases blood pressure through calcium sensitization in VSMCs [260], and that exposure to MMA(III) at high doses induces VSMC dysfunction by impairing voltage-sensitive Ca^{2+} channels and reducing vascular reactivity to contractile agonists. In addition to inducing apoptosis of VSMCs [136,137,261],

arsenic trioxide has been shown to induce apoptosis in human coronary smooth muscle cells [262], microvascular cells [263], human umbilical vein endothelial cells [255], and intestinal endothelial cells [139]. However, some studies have demonstrated a proliferative effect of arsenic on VSMCs [264] and endothelial cells [265], presumably by activating mitogenic signals and inducing the release of vascular endothelium growth factor. Bimodal effects of MMA(III) have also been demonstrated. Lim et al., (2011) reported that MMA(III) elicited higher vasopressor responses at low doses while suppressing vasoconstriction at high doses *in vivo* [138]. Collectively these data suggest that the variable effects of these arsenical species may be caused by differences in concentrations and exposure duration, differences in cell culture conditions, tissue preparations of *ex vivo* models of arsenic toxicity, and cell type analyzed in each study.

As previously mentioned, mitochondria may be a primary target of arsenic in several tissues. Indeed, exposure to arsenic is associated with loss of mitochondrial membrane potential in human pulmonary cell lines [143], and reduced ATP content in rat liver mitochondria [266]. In addition, MMA(III), but not iAs(III) can selectively target and inhibit mitochondrial complexes II and IV in isolated mitochondria from rat liver [250], and induce ROS levels in intact mitochondria in human epithelial cells [139]. The effects of arsenic species on

mitochondrial structure/function and mechanisms of toxicity in VSMCs however, remain to be elucidated.

Our study is the first to comparatively analyze the effects MMA(III) and iAs(III) on mitochondrial structure and function in immortalized rat aortic smooth muscle A7r5 cells, a tissue culture model of VSMCs. The doses of iAs(III) and MMA(III) used in this study are in accordance with environmental exposures of at-risk populations reported in several studies [176,177,267,268] and have been used in previously published studies [27,139,250]. In this study, we report that MMA(III), but not iAs(III), promotes mitochondrial dysfunction, metabolic and morphological alterations, and oxidative stress compared to untreated VSMCs. We also found that MMA(III)-mediated induction of ROS preceded loss of mitochondrial content and cell death. Overall, our data supports a conceptual model that suggests that MMA(III) impairs mitochondrial function by eliciting mitochondrial and non-mitochondrial sources of ROS that contribute to cytopathology of VSMCs.

iii. Materials

Dulbecco's Modified eagle's media (DMEM), trypsin-EDTA solution (0.25% Trypsin, 0.02% EDTA), fetal bovine serum (FBS), antibiotic-antimycotic

(ABAM) (100X), and Amplex® Red Hydrogen Peroxide/ Peroxidase Assay kit were purchased from Invitrogen (Carlsbad, CA). Trypan Blue solution (0.4%), RIPA buffer, inorganic arsenic (As_2O_3) (iAs(III)), 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain, sodium pyruvate solution, sodium hydroxide pellets, methyl iodide, hydrochloric acid, sulfur dioxide, protease inhibitor cocktail, adenosine 5′ -triphosphate (ATP) disodium salt hydrate, dithiothreitol (DTT), catalase from bovine liver, and Bradford Assay Kit were obtained from Sigma-Aldrich (St. Louis, MO). GlutaMax (GIBCO) and MitoSOX (Molecular Probes) were purchased from Thermo Fisher Scientific (Waltham, MA). MnTBAP chloride hydrate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The CellTiter-Glo® Luminescent Cell Viability Assay kit and the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit were obtained from Promega (Madison, WI). The XF Cell Mito Stress Test Kit was purchased from Seahorse Biosciences (Billerica, MA). The broad-spectrum caspase inhibitor Z-VAD-FMK was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY).

The following antibodies were used: mouse anti-OXPHOS (Abcam), rabbit anti- β-tubulin (Abcam), rabbit anti-Tom20 (Santa Cruz Biotech), rabbit anti-cleaved caspase-3 (Cell Signaling Technology), rabbit IgG and mouse IgG

secondary antibodies conjugated to horse radish peroxidase (GE Healthcare Bio-Sciences, Pittsburgh, PA), and Alexa 568-conjugated donkey anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, CA).

Diiodomethylarsine (CH_3AsI_2), a precursor of monomethylarsonous acid (MMA(III)), was synthesized in the Angermann lab as described by Millar et al (1960). In brief, arsenic(III) oxide (As_2O_3) and sodium hydroxide were combined in a solution, to which ethyl alcohol and 1.6M methyl iodide were added. The resulting solution was cooled in an ice bath under continuous stirring for 20h, and evaporated to dryness under vacuum. A fraction of the solid generated was diverted, reconstituted in ethanol, and recrystallized 2x from ethanol as disodium methanearsonate ($Na_2(CH_3AsO_3)$; 'MMA(V)'), which presented as white crystals melting at 161°C. The remainder of the residue was dissolved in water, to which concentrated hydrochloric acid, sulfur dioxide, and potassium iodide (KI) were added, generating a precipitate which was then filtered out, washed twice, and discarded; the lower layer of diiodomethylarsine (the diiodo-salt of MMA(III)) was subsequently separated from the filtrate and recrystallized 2x from methanol. The final product was washed with ice water, vacuum dried, and the resultant yellow crystals were verified as diiodomethylarsine by capillary melting point determination at 30°C [269]. Purity of MMA(III) was

further confirmed via 400 MHz ^{1}H NMR spectroscopy on a Varian 400-MR instrument (**Figure S1**). Diiodomethylarsine crystals were stored at -80°C. MMA(III) working solutions were made fresh as needed by dissolving diiodomethylarsine in ultrapure water, storing at 4°C, and using within 24 hours. Arsenic compounds (iAs(III) and MMA(III)) are carcinogens and skin irritants. Health Precautions: Proper precautions should be taken to avoid inhalation or direct skin contact by wearing proper laboratory attire including a laboratory coat, safety goggles, and nitrile gloves when handling these compounds.

iv. Methods

iv.i. Vascular smooth muscle cell cultures

Immortalized rat aortic smooth muscle cells (A7r5) were generously provided by Dr. Dean Burkin (Department of Pharmacology, University of Nevada School of Medicine). Cells were seeded in polystyrene tissue culture treated T-75 flasks (Sigma-Aldrich) in DMEM containing 4500 mg/L glucose and 100 mg/mL sodium pyruvate supplemented with 10% FBS and 1% ABAM. Cells were maintained at 37°C / 5% CO_2 and passaged at 80% confluency. All experiments were conducted on cells derived from passages between 5 and 25.

iv.ii. Bright field microscopy

A7r5 cells were seeded at 30,000 cells per well in tissue culture treated 24-well plates and adhered for 24 hours prior to treatment with increasing concentrations of iAs(III) (0.1μM, 1μM, 10μM) or MMA(III) (0.1μM, 1μM , 10μM). Live cells were imaged under bright field phase for morphological changes 24 hours post exposure to arsenic species at a magnification of 10X or 20X on an AxioVert 200M microscope (Zeiss) or with an EVOS-FL microscope at the same magnification (Life Technologies).

iv.iii. Trypan Blue exclusion assay for cellular mortality

Cells were plated at 30,000 cells per well in a 24-well plate for 24 hours, after which cells were treated with increasing concentrations of iAs(III) or MMA(III) for 24 hours. Cells were then trypsinized with 0.25% trypsin to obtain aliquots of cell suspension. Active measurements of cell death induced by arsenic toxicity were conducted by manually counting aliquots of cell suspension by using a 1:1 dilution of Trypan Blue Exclusion dye. The lethal concentrations at 50% (LC50) for MMA(III) and iAs(III) at 24 h were determined using Origin software (version 6.0). To complement our Trypan Blue Exclusion assays, cell viability was determined by performing cell counts of cell suspensions of A7r5

cells treated with increasing concentrations of arsenicals by using a Z™ series-Coulter Counter (Beckman Coulter) (**Figure S3**).

iv.iv. Cell survival assays

To measure the effects of iAs(III) and MMA(III) on cell viability of VSMCs, cells were seeded in opaque-walled, clear bottom 96 well plates at 10,000 cells per well. Twenty-four hours after seeding, cells were treated with either 0.5μM MMA(III) or 10μM iAs(III) for 24 hours, with or without the broad spectrum caspase inhibitor Z-VAD-FMK at 40μM, followed by incubation with the CellTiter 96® AQueous One Solution MTS reagent for 1hour per manufacturer's instructions. Absorbance was measured at 490 nm with a Multi-mode SpectraMax M4 96 well plate reader (Molecular Devices, Sunnyvale, CA). It is important to note that the amount of iodide present in MMA(III) stocks does not significantly affect cell viability compared to water-treated cells or untreated cells as determined by performing the MTS assay (**Figure S2A**) or DAPI counts (data not shown), nor does it impact mitochondrial content (data not shown). Hence, all ensuing survival assays were done in vehicle-treated cells compared to MMA(III)-treated cells.

iv.v. Mitochondrial respiration assay

Cellular respiration was analyzed in cells seeded in a XF24 V7 Cell Culture Microplate by using an XFe24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA). In brief, A7r5 cells were plated at a density of 10,000 cells per well for 24 hours prior to analysis of mitochondrial respiration. The oxygen consumption rates (OCRs) and extracellular acidification rates (ECARs) were measured in quadruplicate wells for basal respiration by performing four measuring cycles (~30 minutes) followed by injecting the wells with vehicle or arsenic compounds to analyze for toxic effects of arsenic species on mitochondrial respiration. Cells were then sequentially treated with 1μM oligomycin to measure ATP-linked mitochondrial respiration, 1μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) to measure the maximal respiratory capacity, and with 1μM rotenone/antimycin-A to measure mitochondria-specific respiration as previously described [270,271]. We also performed concentration-dependent studies of MMA(III) to measure the lowest dose at which MMA(III) induces a deficit in mitochondrial respiration in A7r5 cells. Following each assay, OCRs and ECARs were normalized to protein concentration by using the Bradford Assay. The line trace of the oxygraph for each experiment was plotted and the mean values of OCRs and ECARs with

standard errors of the mean (SEM) were calculated by using the WAVE 2.0 software (Seahorse Biosciences). All cells not exposed to MMA(III) were treated with water. However, it is worth noting that the iodide contained in stocks of MMA(III) did not significantly contribute to a decrease in mitochondrial respiration, suggesting negligible potential for MMA(III) solution-derived iodide toxicity in this model (**Figure S2B**).

iv.vi. Intracellular detection of hydrogen peroxide levels

A7r5 cells were seeded at 30,000 cells per well in 6-well plates for 24 hours prior to analysis of extracellular levels of hydrogen peroxide by using the Amplex Red kit. On the day of the assay, the media was replaced with fresh media containing a dilution of 1:1000 Amplex Red, 1:100 horseradish peroxidase (HRP), and iAs(III) (1μM) or MMA(III) (100nM, 1μM, 10μM) and incubated at 37 °C for 2 hours. A Tecan Ultra microplate reader (Männedorf, Switzerland) was used to measure absorption and fluorescence emission at 530 nm and 590 nm, respectively. The data was plotted as the mean fluorescence derived from at least 5 wells per condition.

iv.vii. Mitochondrial superoxide assays

To measure the effect of arsenic species on the levels of mitochondria-derived superoxide at different time points, cells were stained with MitoSOX (Molecular Probes) as described previously [270,272]. As a control for induction of mitochondrial superoxide, cells were treated with 1μM rotenone for 2 or 4 hours. We used Image J (version 1.44, Bethesda, MD) to calculate the mean fluorescence intensity of MitoSOX per cell for at least 30 cells per condition as previously described [270,271].

ii.viii. Western blot analysis

Cell lysates were prepared by harvesting cells in lysis buffer (1.0% Triton X-100 with phosphatase inhibitors sodium orthovanadate and sodium pyrophosphate, and protease inhibitors E64 and PMSF). Twenty-five micrograms of cell lysates were resolved on 10% SDS-PAGE Tris-buffered polyacrylamide gels by using the mini-gel BioRad system as previously described [273]. At the end of the SDS-PAGE run, the proteins were transferred onto methanol pre-activated PVDF membranes by using the Transblot SD Semi-Dry Transfer electrophoresis system (BioRad, Hercules, CA). The PVDF membrane was then blocked in either 2% BSA or 5% non-fat dry milk for 1 hour, washed in phosphate buffered saline with

0.1% Triton X-100 (PBST), and incubated overnight at 4°C with primary antibodies specific for the outer mitochondrial membrane (OMM) -localized protein TOM 20 (1:2000), for subunits of various mitochondrial-encoded mitochondrial complexes (OXPHOS) (1:2000), and for β-tubulin (1:10,000) as a loading control. PVDF membranes were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (1:5,000) in PBST and immunoreactive bands were imaged by chemiluminescence by using a BioRad Imager available at the Nevada Proteomics Center.

iv.ix. Immunofluorescence

To image mitochondria, VSMCs were fixed in 4% paraformaldehyde, permeabilized in PBST containing 0.1% Triton X-100 and stained for mitochondria by exposing cells to rabbit-anti-human TOM 20 antibody overnight at 4°C. The cells were then washed extensively in PBST and incubated with Alexa 568-conjugated donkey anti-rabbit IgG (1:1,000), and counterstained with 1.25 μg/ml DAPI to stain for nuclei. Immunolabeled cells were imaged at 25°C by using an EVOS-FL Cell Imaging System equipped with EVOS Light cubes specific for GFP (Ex/Em of 470/510), RFP (Ex/Em of 531/593) and Cy5 (Ex/Em of

628/692) and at a magnification of 20X (0.45NA) or 40X (0.60NA). NA: numerical aperture.

iv.x. Fluorescence-based image analyses of cell density and active cell death

To determine whether MMA(III) induces caspase-3 mediated apoptosis, we assessed cell density and cell death in VSMCs that were treated with LD50 concentrations of arsenicals by performing image analyses of caspase-3 and DAPI-stained cells. To assess cell density, the number of nuclei per epifluorescence micrograph was manually counted blind to the observer for at least 300 cells per condition. By using the semi-automated Apoptosis Cell Density Image J macro, (http://imagejdocu.tudor.lu/doku.php?id=plugin:morphology:apoptosis_and_cell_count_macro:start), we also analyzed for cell death induced by arsenicals by counting the percentage of cells containing fragmented, pyknotic, or irregular-shaped nuclei per epifluorescence micrograph, morphological hallmarks of cell death (**Figure S4**). Arsenical-treated cells were also stained for cleaved caspase-3 to determine the extent by which MMA(III) or iAs(III) promote late stage apoptosis in A7r5 cells. Immunolabeled cells were imaged at 25°C by using the EVOS-FL Cell Imaging System as described above.

The percentage of cells that stained for caspase-3 per epifluorescence micrograph was determined to assess the ability of MMA(III) or iAs(III) to induce apoptosis.

iv.xi. Mitochondrial morphology

To analyze the effects of arsenic species on mitochondrial morphology 5 hours post treatment, the average area/perimeter ratio per cell was measured as an index of mitochondrial interconnectivity in arsenic-treated VSMCs by using the "Mitochondrial Morphology Macro" (available for free on the Wiki site http://imagejdocu.tudor.lu/) on Image J software (v1.39) as previously described [274]. We chose 5 hours as this is the longest duration of treatment with iAs(III) or MMA(III) for functional and non-cytotoxic assays done in this study.

iv.xii. ATP assay

Mitochondrial ATP concentrations were measured by using the CellTiter-Glo^{R} Luminescent Cell Viability Assay (Promega, USA) and a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA) as previously described [270,271]. In brief, cells were plated at 10,000 cells per well in opaque-walled 96-well microplates and cultured in DMEM containing 10mM galactose, 2mM glutamine, 2mM sodium pyruvate and 10% FBS for 24 hours prior to treatment

with arsenic. Under these media conditions, the cells predominantly rely on mitochondrial oxidative phosphorylation for ATP production [275]. Cells were exposed to iAs(III) (1μM, 10μM) or MMA(III) (0.5μM, 1μM) for 6 hours. To assess for mitochondrial ATP levels, one half of the wells containing cells were treated with the ATP synthase inhibitor oligomycin for 1hour at 1μM. The luminescence readings were converted to ATP concentrations (μM) based on a 0 -10μM standard curve and normalized to protein concentration by using the Bradford assay. Mitochondrial and cytosolic (non-mitochondrial) ATP levels were determined based on the following equation: Mitochondrial steady-state ATP levels= (Total cellular steady-state ATP levels) – (oligomycin-resistant steady state ATP levels, cytosolic ATP). For some experiments, cells were cultured in high glucose media to induce glycolysis to assess the effects of iAs(III) or MMA(III) on cytosolic ATP levels in glycolytic conditions.

iv.xiii. Toxicity reversal studies with antioxidants

To determine whether mitochondrial superoxide and/or hydrogen peroxide play a role in arsenic-mediated mitochondrial dysfunction, A7r5 cells were plated at 10,000 cells per well in a XF24 V7 Microplate and either pre-treated with manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) -a

superoxide dismutase mimetic- at 100μM for 24 hours prior to the addition of MMA(III) (0.5μM, 2hours), or co-treated with 10 units/ml of catalase for the duration of MMA(III) treatment (0.5μM, 2hours) to scavenge hydrogen peroxide. This specific dose of MMA(III) consistently decreases mitochondrial respiration (**Figure 6**). Following treatments with anti-oxidants, the baseline, maximal, and ATP-linked mitochondrial respiration were measured as described above (*Mitochondrial respiration assay* section). It is worth noting that the brown color in the media formed in cells co-treated with MnTBAP impaired the ability of the XF24e Analyzer to read OCRs, which precluded an analysis of the effects of co-treating cells with MnTBAP and arsenic compounds on mitochondrial function. Hence, for this reason, it was necessary to pretreat (not co-treat) VSMCs with MnTBAP for 24 hours prior to reading OCRs in arsenic-treated cells.

To study the effects of antioxidants on arsenic-induced cell death, cells were plated in opaque-walled 96-well plates at 10,000 cells per well. Following an adherence time of 24 hours, cells were co-treated with 0.5μM MMA(III) or 10μM iAs(III) in the presence of either 100μM of MnTBAP or 10 units/ml of catalase for 24 hours. Following a 24 hour exposure, cells were incubated with One Solution MTS reagent for 1 hour and absorbance was measured at 490 nm by using a Multi-mode SpectraMax M4 96 well plate reader as described above.

The absorbance of wells containing cells and treatments were subtracted from wells lacking cells but containing the respective backgrounds. It is worth noting that cells were treated with the above concentrations of arsenicals for 24 hours in order to elicit a consistent, measurable and significantly consistent decrease in cell viability (~50% cell death) prior to assessing for potential significant reversal of cytotoxicity with antioxidants.

iv.xiv. Statistical analysis

All statistical analyses were performed by using the SPSS software (IBM) (v 23). Unless indicated otherwise, most experiments were performed at least three times and the results are presented as means ± standard errors (SE). By applying an α of 0.05, One-Way ANOVAs followed by the *post hoc* Tukey's HSD tests were conducted for all experiments to account for multiple comparisons in data interpretation.

v. Results

v.i. Viability of A7r5 Cells following exposure to iAs(III) and MMA(III)

We conducted several cell survival assays to compare the cytotoxic potentials of iAs(III) and MMA(III) in VSMCs at 24 hours. Qualitative analyses of

cell morphology revealed that exposure of cells to iAs(III) induced morphological changes associated with necrosis while MMA(III) induced morphological changes consistent with apoptosis (pyknosis and cell blebbing) (**Figure 1A**). Consistent with the observation that MMA(III) promotes apoptosis, we observed that treating cells with MMA(III), but not iAs(III), robustly elicited the activation of caspase-3 and nuclear fragmentation, pathological hallmarks of apoptosis (**Figure S4A-B**). Furthermore, co-treating cells with the pan-caspase inhibitor Z-VAD-FMK conferred partial protection against MMA(III)-mediated cell death suggesting that factors other than caspases also contribute to cell death (**Figure S4C-D**). This data is in agreement with other reported studies that show that MMA(III) and iAs(III)-mediated cell toxicity is associated with the activation of apoptosis and necrosis respectively [138-140]. The LC50s at 24 hours for MMA(III) and iAs(III) were determined to be 0.50μM and 10.0μM respectively as assessed by three independent methods: the trypan blue exclusion assays (**Figure 1B,C**), and further corroborated by the MTS assay, a more quantitative and non-biased method for assessing cell viability (**Figure 1D**), and by performing cell counts using a Coulter counter apparatus (**Figure S3**). Collectively, these data show that MMA(III) is 20x more toxic than iAs(III) to VSMCs at 24 hours. Based on these cell death studies, all subsequent mitochondrial function and

morphological analyses were done using sublethal exposures of arsenical that do not result in significant cell death (**Figure S4**).

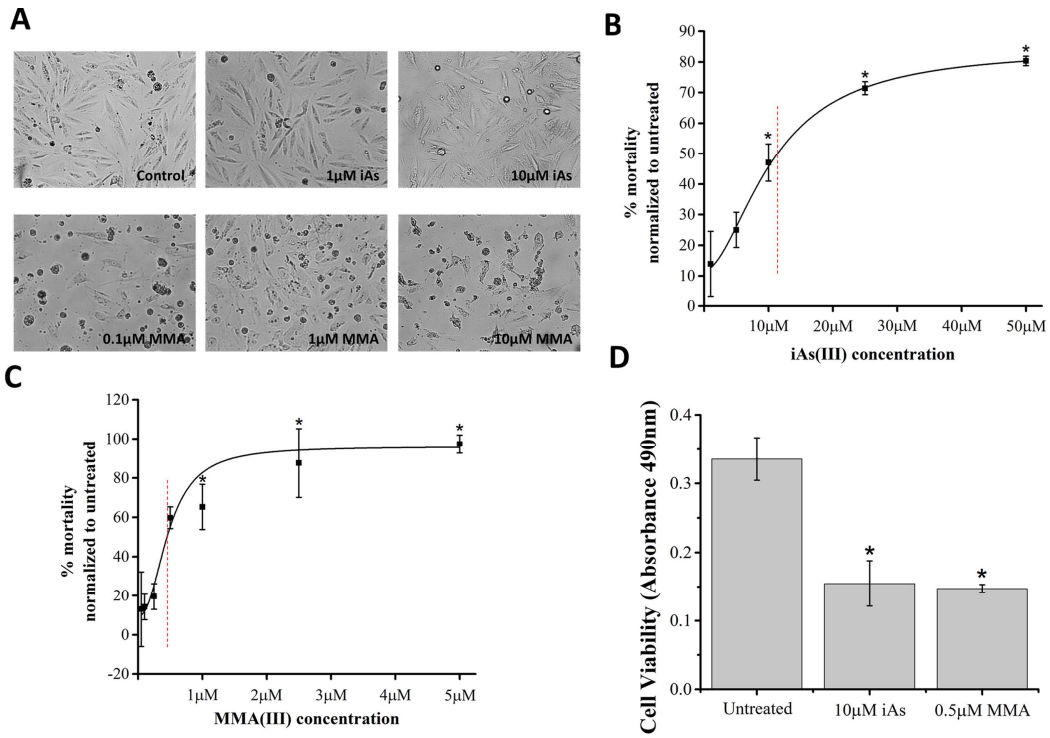


Figure 1. Differential morphological and toxicological effects of MMA(III) and iAs(III) in A7r5 cells.

A. Representative images of live A7r5 cells treated with the indicated concentrations of toxicants or vehicle control (Control) for 24 hours. Cells were imaged with an AxioVision (Zeiss) microscope at 10X magnification. Representative micrographs of cells with MMA(III) show pyknosis and blebbing, consistent with apoptosis.

B, C. Cell mortality curves were assessed by performing the Trypan Blue Exclusion Dye assay for cells treated with iAs (panel B) or with MMA(III) (panel C). The percent mortality was calculated as follows: [Cell mortality= Trypan Blue stained cells/total cells * 100]. The 24 hour lethal concentrations at 50% (LC50s) were determined to be 0.5μM for MMA(III) and 11.3μM for iAs(III) as indicated by red-hatched lines. Dose dependent graphs are representative of three experiments (*:$p<0.05$ vs. untreated, Means ±SE, n=3 samples per condition). One-way ANOVA, Tukey's HSD.

D. Viability of A7r5 cells was assessed via the MTS assay following 24 hour exposure. Cells treated with 0.5μM MMA(III) or with 10μM iAs(III) showed a significant decrease in cell viability

(*:p<0.05 vs. untreated, Means±SE, n=18 wells/condition). One-way ANOVA, Tukey's HSD. Data was compiled from 3 independent experiments.

v.ii. Effects of iAs(III) and MMA(III) on mitochondrial respiration

We measured the effects of both inorganic and organic arsenic species on mitochondrial function by using an Extracellular Flux XF24[e] Analyzer, a well-characterized system for measuring real-time oxygen consumption in live cells. In brief, we found that prelethal exposure of cells to 0.5μM of MMA(III), but not iAs(III) at 10μM, progressively induced a significant decrease in the basal respiration of VSMCs within 3 hours of treatment (**Figure 2A,B**). For comparative purposes, other OCR traces show consistency and little variability in the temporal dynamics of MMA(III)-mediated decrease in OCRs across experiments (**Figure S5**). It is worth noting that treating cells with MMA(III), but not with iAs(III), led to a progressive reduction in baseline OCR that plateaued by 100 min. of treatment (**Figure 2A, Figure S5**). In addition, we observed a significant decrease in maximal oxygen consumption rates (OCRs) in cells exposed to 0.5μM MMA(III), but not with 10μM iAs(III) (**Figure 2 A, Fig. S6**). MMA(III) is a mitochondria-specific toxicant since treating cells with MMA(V) at similar concentrations (1μM) did not decrease OCRs in A7r5 cells (**Figure S6**).

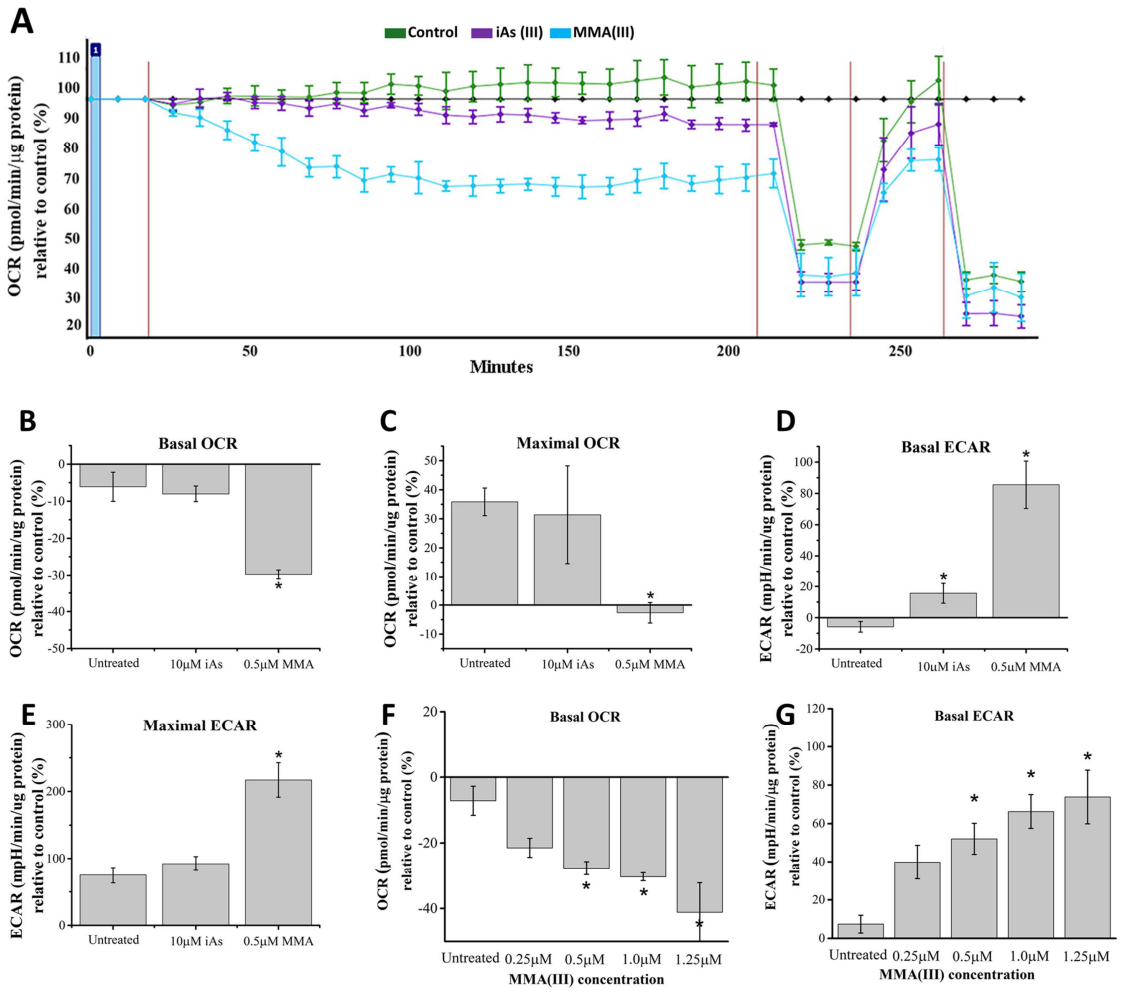


Figure 2. Effect of arsenic species on mitochondrial respiration.

A. Representative mitochondrial respiration profile (oxygraph) of three experiments demonstrates that VSMCs treated with 0.5µM MMA(III), but not with 10µM iAs(III) show a progressive decrease in baseline OCRs followed by a reduction in maximal OCRs compared to control (untreated). The oxygraph is representative of three experiments with similar results and all other traces have been uploaded as supplemental information (**Figure S5**).

B. Comparative analysis of basal respiration, measured as a percentage relative to time 0, demonstrates that VSMCs treated with 0.5µM MMA(III) for 3 hours showed a significant decrease in baseline OCRs relative to untreated cells or cells treated with 10µM iAs(III) (*:$p<0.05$ vs. untreated cells, **:$p<0.05$ vs. 10µM iAs(III) treated cells, Means, ±SE, n=10-16 wells/condition). One-way ANOVA, Tukey's HSD. Data was compiled from three independent experiments.

C . Comparative analysis of maximal mitochondrial respiration, measured as a percentage relative to time 0, demonstrates that VSMCs exposed for 3 hours to 0.5µM MMA(III) show

significantly reduced maximal OCRs compared to untreated cells or cells treated with 10μM iAs(III). To measure maximal mitochondrial respiration, cells were treated with 1μM FCCP and OCRs were measured for three consecutive time points (*:p<0.05 vs. untreated cells,**:p<0.05 vs. 10μM iAs(III) treated cells, graph shows means, ±SE, n=10-16 wells/condition). One-way ANOVA, Tukey's HSD. Data was compiled from three independent experiments.

D. Comparative analyses of extracellular acidification rate (ECAR) show that treating VSMCs with 0.5μM MMA(III) or with 10μM iAs(III) for 3 hours leads to a significant increase in ECARs (*:p<0.05 vs. untreated cells, Means, ±SE, n=10-16 wells/condition). Treatment with 0.5μM MMA(III) resulted in a non-significant increase in ECAR relative to treatment with 10μM iAs(III). One-way ANOVA, Tukey's HSD. Data was compiled from three independent experiments.

E. Comparative analyses of maximal ECARs show that treating VSMCs with 0.5μM MMA(III) leads to a significant increase in maximal ECARs compared to untreated cells or cells treated with 10μM iAs(III). (*:p<0.05 vs. untreated cells, **:p<0.05 vs. 10μM iAs(III) treated cells, Means, ±SE, n=10-16 wells/condition). One-way ANOVA, Tukey's HSD. Data was compiled from three independent experiments.

F. Dose-dependent analyses show that baseline OCRs were significantly reduced in VSMCs treated with MMA(III) in a dose dependent manner. (*:p<0.05 vs. untreated, Means, ±SE n=8-14 wells/condition). One-way ANOVA, Tukey's HSD. Data was compiled from three independent experiments.

G. Dose-dependent analyses show that baseline ECARs were significantly reduced in VSMCs treated with MMA(III) in a dose dependent manner (*:p<0.05 vs. untreated, Means, ±SE n=8-14 wells/condition). One-way ANOVA, Bonferroni adjusted Tukey's HSD. Data was compiled from three experiments.

Interestingly, treating VSMCs with MMA(III) led to a significant increase in basal and maximal extracellular acidification rates (ECARs), a proxy of glycolysis. This data suggests that MMA(III) toxicity leads to a compensatory increase in glycolysis when mitochondrial function is impaired, also known as the Warburg effect [276] (**Figure 2D,E**). Treatment with iAs(III) led to a robust increase in basal ECARs (**Figure 2D**) but maximal ECARs were not significantly affected (**Figure 2E**), suggesting that treatment of VSMCs with iAs(III) leads to a

partial upregulation of ECAR. Next, we wanted to assess the minimal concentration of MMA(III) that can induce alterations in mitochondrial respiration and glycolysis in VSMCs. Dose-dependent analysis of OCRs and ECARs revealed that MMA(III) can induce a significant decrease in basal OCRs and ECARs at concentrations as low as 0.5μM. A non-significant decrease in OCRs was observed in cells exposed to 0.25μM of MMA(III). (**Figure 2F, G**). Overall, these data suggest that treating A7r5 cells with sublethal doses of MMA(III), but not iAs(III), selectively impair mitochondrial respiration while concomitantly upregulating glycolysis, possibly as a protective mechanism to compensate for mitochondrial dysfunction.

v.iii. Effects of Arsenic compounds on mitochondrial ATP levels

Given that oxidative phosphorylation drives the synthesis of mitochondrial ATP, we then investigated whether MMA(III)-mediated deficits on mitochondrial respiration are associated with a decline in mitochondrial ATP levels. We observed that exposing VSMCs to 0.5μM MMA(III) for 6 hours led to a significant decrease in mitochondrial ATP levels, while 1μM MMA(III) significantly decreased cytosolic ATP levels (**Figure 3**). To further corroborate the effects of MMA(III) on cytosolic ATP steady-state levels, we observed that

prelethal exposure to MMA(III), but not iAs(III), contributed to a modest but significant decrease in cytosolic ATP levels in cells grown in high glucose-containing media (Control: 2.98μM ±0.12; MMA(III): 2.21 μM ±0.06, $p<0.00001$). On the other hand, treatment with 1μM or 10μM iAs(III) had no significant effect on either mitochondrial, cytosolic, or total ATP levels compared to control cells in no-glucose media (**Figure 3**) or under glycolytic conditions (Control: 2.98μM ±0.12; iAs(III): 2.43 μM ±0.18, $p=0.25$). It is worth noting that treating cells with these doses of arsenicals for 6 hours does not cause significant cell death which rules out the possibility that any observed decreases in ATP levels is due to cytotoxicity (**Figure S4**). These results suggest that MMA(III) significantly decreases mitochondrial respiration and ATP levels while iAs(III) does not.

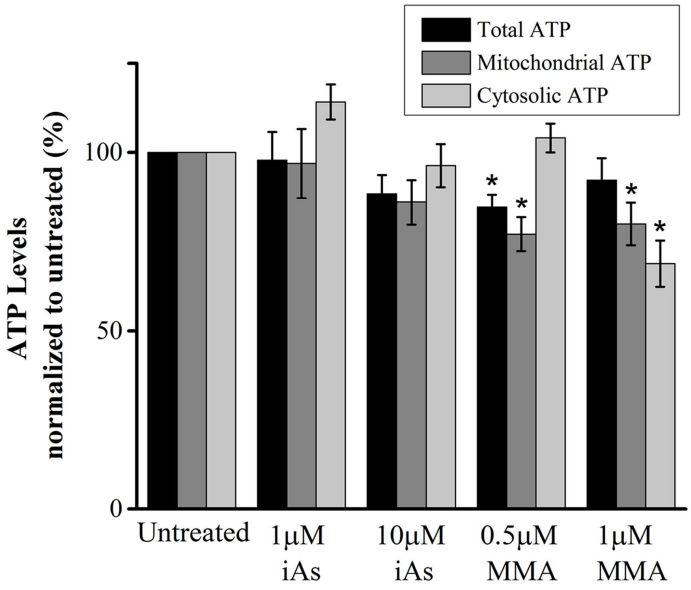


Figure 3. Effect of arsenic species on mitochondrial ATP levels.

Compiled quantification of ATP levels in VSMCs treated with the indicated concentrations of MMA(III) and iAs(III) for 6 hours. Mitochondrial ATP levels were determined by subtracting the levels of ATP not affected by electron chain complex inhibitors rotenone and antimycin-A (cytosolic ATP) from total ATP levels. Total, mitochondrial or cytosolic ATP levels shown in the compiled quantification was normalized to its respective untreated control within each group (total, mitochondrial or cytosolic).Treatment with 0.5μM MMA(III) resulted in a significant decrease in mitochondrial ATP levels relative to untreated cells, while treatment with 1μM MMA(III) resulted in a significant decrease in cytosolic ATP relative to untreated cells. (*:$p<0.05$ vs. untreated cells, Means, ±SE, n=18 wells/condition) One-way ANOVA, Tukey's HDS. Data was compiled from three independent experiments.
Please note that in media conditions that do not have glucose but is supplemented with pyruvate and galactose, we observe that cells produce 50-60% of ATP from mitochondria whereas cells grown in high glucose media, which stimulate glycolysis, predominantly produce non-mitochondrial (cytosolic) ATP (~80-90% of total ATP).

v.iv. Effects of arsenic compounds on ROS levels

Given that MMA(III) can significantly suppress mitochondrial respiration, we then wanted to identify mechanisms of MMA(III)-mediated mitochondrial dysfunction in VSMCs. Arsenic compounds are known to stimulate the production of hydrogen peroxide in myriad cell types [277,278]. Hence, we surmised that an elevation in the levels of hydrogen peroxide may be correlated with mitochondrial dysfunction induced by MMA(III). In brief, we indirectly measured the levels of hydrogen peroxide in cells exposed to arsenic species by using the Amplex Red assay. We observed that increasing concentrations of MMA(III) induced a significant elevation of hydrogen peroxide levels compared to control cells or iAs(III) at 2 hours post exposure. This data suggests that

elevated hydrogen peroxide levels induced by MMA(III) may contribute to mitochondrial and cellular pathology (**Figure 4A**).

v.v. Generation of Superoxide

Arsenic toxicity has been associated with increased levels of mitochondrial superoxide in other cell lines [250]. Given that hydrogen peroxide is derived from mitochondrial superoxide, we investigated the extent to which arsenic species can induce the production of mitochondrial superoxide by staining live cells with the red-fluorescent, cell permeable superoxide indicator MitoSOX. Image analyses of mean MitoSOX fluorescence suggests that treating cells with 0.5μM or 1μM MMA(III) can significantly elevate mitochondrial superoxide levels at 4 hours compared to control cells or cells treated with 1μM or 10μM iAs(III) (**Figure 4B**). MMA(III)-mediated elevation in superoxide is commensurate with oxidative stress induced by exposure to the complex I inhibitor rotenone. Neither arsenic species significantly elevated mitochondrial superoxide levels at 2 hours, while only a non-significant increase in superoxide was induced by iAs(III) at 4 hours (**Figure 4B**). However, at longer exposures of 6 hours, we found that iAs(III) can significantly increase mitochondrial superoxide levels compared to untreated cells (**Figure S8**). Our data is consistent with

another study that showed a stronger induction of ROS in MMA(III) compared to iAs(III) in liver cells [250]. Collectively, this data suggests that MMA(III) is a more efficient inducer of mitochondrial superoxide compared iAs(III) in VSMCs.

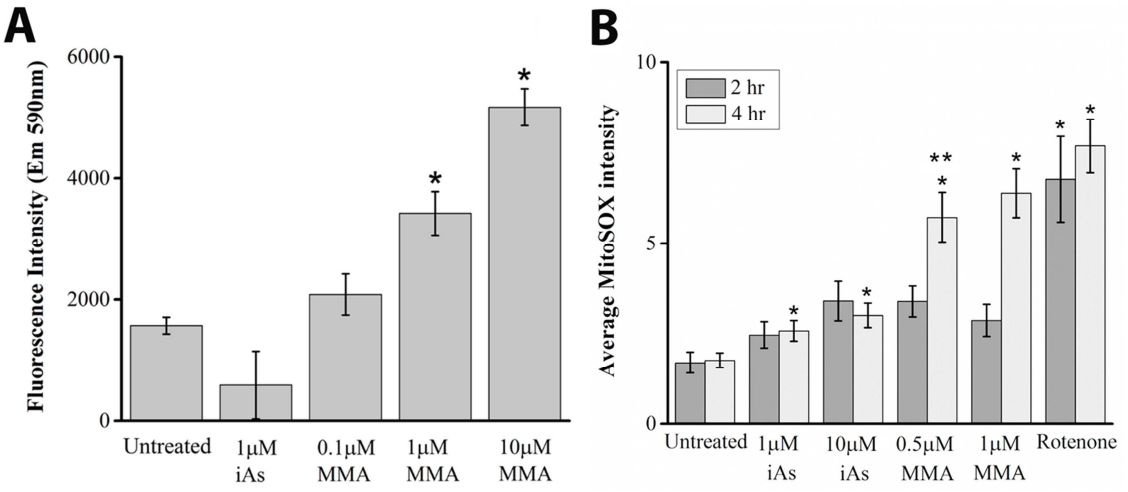


Figure 4. Effects of arsenic species on hydrogen peroxide and mitochondrial superoxide levels.

A. Mean hydrogen peroxide levels, as assessed by the Amplex red assay, were significantly elevated in cells exposed to 1μM or 1μM MMA(III) for 2 hours compared to untreated cells or cells treated with 1μM iAs(III) (*:$p<0.05$ vs. untreated cells, **:$p<0.05$ vs. 1μM iAs(III) treated cells, graph shows means, ±SE, n=4 wells/condition). One-way ANOVA, Tukey's HSD. Data is representative of one of three experiments.

B. The average intensity of MitoSOX fluorescence was quantified in untreated cells, and in cells exposed for 2 or 4 hours to the indicated concentrations of MMA(III), iAs(III) or with the complex I inhibitor rotenone as a positive control for mitochondrial superoxide induction. Mitochondrial superoxide was significantly elevated among cells treated for 4 hours with 0.5μM or 1μM MMA(III) compared to untreated cells, or cells treated with iAs(III) at a concentration of 1μM or 10μM (*:$p<0.05$ vs. untreated, **:$p<0.05$ vs. iAs(III), Means, ±SE, n=25-46 cells/condition). One way ANOVA, Tukey's HSD. Data was compiled from three independent experiments.

v.vi. Effects of Arsenic compounds on mitochondrial morphology and content

Next, we surmised that the decreased mitochondrial respiration and ATP levels induced by MMA(III) leads to a decrease in mitochondrial content in VSMCs. To address this hypothesis, we quantified mitochondrial content in VSMCs by using image-based analysis of mitochondrial content in VSMCs immunostained for mitochondria and by immunoblotting for the total cellular levels of various mitochondrial markers. Western blot analyses of mitochondrial markers revealed that treating VSMCs with 1μM MMA(III) for 5 hours, a concentration that is sufficient to induce mitochondrial damage and oxidative stress but not cell death (**Figure 2, 4 and S4**), significantly decreased the levels of mitochondrial markers localized either to the outer mitochondrial membrane (TOM20) or the inner mitochondrial membrane (ATP synthase V) (**Figure 5A-C**). On the other hand, treating cells with 1μM iAs(III) for 5 hours caused a non-significant decrease in mitochondrial protein levels (**Figure 5A-C**). This data is consistent with the effects of MMA(III) on mitochondria-derived ATP levels (**Figure 3**). In further agreement with our Western blot data, image analyses of VSMCs immunostained for mitochondria revealed that cells treated with 1μM MMA(III) or 1μM iAs(III) for 5 hours led to a significant decrease in mitochondrial content (% of cytosol occupied by mitochondria) (**Figure 5D**).

Given that mitochondrial content is regulated by mitochondrial turnover through the autophagosomal-lysosomal pathway (mitophagy) and the fact that mitochondrial fragmentation precedes mitophagy [279-281], we then surmised that treating cells with both arsenic species causes mitochondrial fragmentation. Unexpectedly, we observed that 1μM MMA(III), but not 1μM iAs(III), promoted perinuclear clustering of mitochondria and a significant increase in mitochondrial interconnectivity at 5 hours post exposure (**Figure 5 E-F**). However, treatment of cells with longer exposure of 10μM iAs(III) can decrease mitochondrial content eventually and fragment mitochondria (**Figure S9**). Overall, our immunofluorescence data highlights significant differences in the ability of MMA(III) and iAs(III) for altering mitochondrial morphology and mitochondrial distribution.

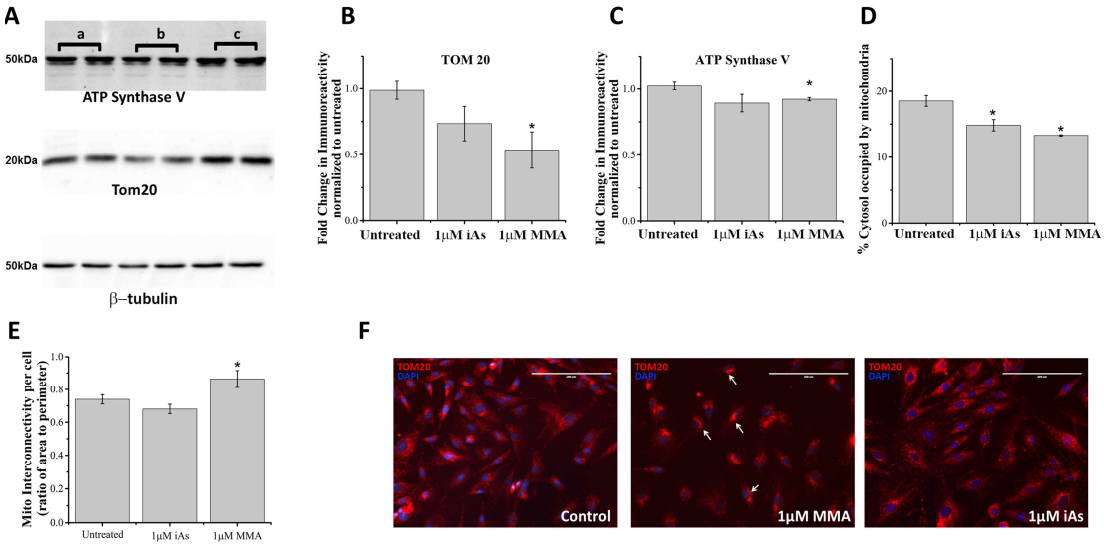


Figure 5. Effects of arsenic species on mitochondrial content and morphology.

A. Representative Western blot analyses of the total cellular levels of mitochondrial markers (TOM20 and ATP synthase V) and of β-tubulin in cell lysates from untreated cells (lanes indicated by **a**), and cells exposed for 5 hours to 1μM MMA(III) (lanes indicated by **b**) or exposed to 1μM iAs(III) (lanes indicated by **c**).

B. Densitometric analyses of the immunoreactivity of the outer membrane localized protein TOM20 as assessed by Western blot of cell lysates from cells treated with indicated concentrations of MMA(III) and iAs(III) and normalized to untreated cells. (*:$p<0.05$ vs. untreated, Means, ±SE, n=5 lanes/condition). One-way ANOVA, Tukey's HSD. Data was compiled from three independent Western blot experiments.

C. Densitometric analyses of the immunoreactivity of the inner membrane localized protein ATP Synthase V as assessed by Western blot of cell lysates derived from cells treated with indicated concentrations of MMA(III) and iAs(III) and normalized to untreated cells. (*:$p<0.05$ vs. untreated, Means, ±SE, n=5 lanes/condition). One-way ANOVA, Tukey's HSD. Data was compiled from three independent Western blot experiments.

D. Image-based quantification of mitochondrial content in paraformaldehyde-fixed cells immunolabeled for TOM20 as assessed by measuring the percentage of the cytosolic area occupied by fluorescently-labeled mitochondria-specific pixels and normalized to untreated cells. (*:$p<0.05$ vs. untreated cells, Means, ±SE, n=44-57 cells/condition). One-way ANOVA, Fishers LSD Tukey's HSD. Data was compiled from three independent experiments.

E. Mitochondrial interconnectivity, defined as the ratio of the average area to the perimeter of mitochondria per cell (Dagda et al. 2009), was measured in untreated cells and in cells treated with the indicated concentrations of iAs(III) and MMA(III). Cells treated with MMA(III) had significantly increased interconnectivity compared to untreated cells or cells treated with iAs(III)

(*:p<0.05 vs. untreated, **:p<.05 vs. 1μM iAs(III), Means, ±SE, n=44-57 cells/condition). One-way ANOVA, Fishers LSD Tukey's HSD. Data was compiled from three independent experiments.

F. Representative epifluorescence micrographs of paraformaldehyde-fixed cells immunolabeled for TOM20 (red) and counterstained with DAPI to visualize mitochondria and nuclei respectively. Epifluorescence micrographs were acquired at a magnification of 20X. While untreated VSMCs showed a normal mitochondrial morphology (tubular to interconnected), cells treated with MMA(III), but not iAs(III), showed the presence of aberrant mitochondrial clusters (indicated by white arrows). Data is representative of one of three experiments with similar results.

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v.vii. Mitochondrial and non-mitochondrial sources of ROS contribute to arsenic toxicity in VSMCs

Given that MMA(III), but not iAs(III), selectively promotes mitochondrial dysfunction and is a more efficient inducer of ROS than iAs(III), we then surmised that mitochondrial superoxide and hydrogen peroxide induced by MMA(III) contributes to mitochondrial dysfunction in VSMCs. To address this hypothesis, we co-treated VSMCs with catalase or pretreated them with the superoxide dismutase mimetic MnTBAP- two anti-oxidants that can scavenge hydrogen peroxide and mitochondrial superoxide, respectively. Following exposure to antioxidants, VSMCs were treated with 0.5μM MMA(III) and assayed for mitochondrial respiration by employing a XF24^{e} Extracellular Flux Analyzer. We observed that MnTBAP treatment partially but significantly ameliorated MMA(III)-mediated deficits in basal OCRs but was unable to reverse the decrease in maximal OCRs (**Figure 6B-D**). Interestingly, MnTBAP treatment

was able to reverse the increase in maximal ECARs induced by MMA(III) (**Figure 6F)**. Treating VSMCs with catalase, on the other hand, completely reversed MMA(III)-mediated decreases in basal and maximal OCRs and was able to block the increase in ECARs (**Figure 6A, C-F**). Next, we generated "phenograms" - visual representations of the cell's energy profile derived by plotting OCRs vs. ECARs- to further understand the metabolic rescue mediated by catalase and MnTBAP treatment in cells exposed to MMA(III). We observed that treating cells with catalase not only reversed mitochondrial dysfunction induced by MMA(III), but it also significantly reversed the energy profile of VSMCs back to a more aerobically active and less glycolytic phenotype compared to MMA(III)-treated cells (**Figure 7A**) whereas treating VSMCs with MnTBAP had a partial effect (**Figure 7B**).

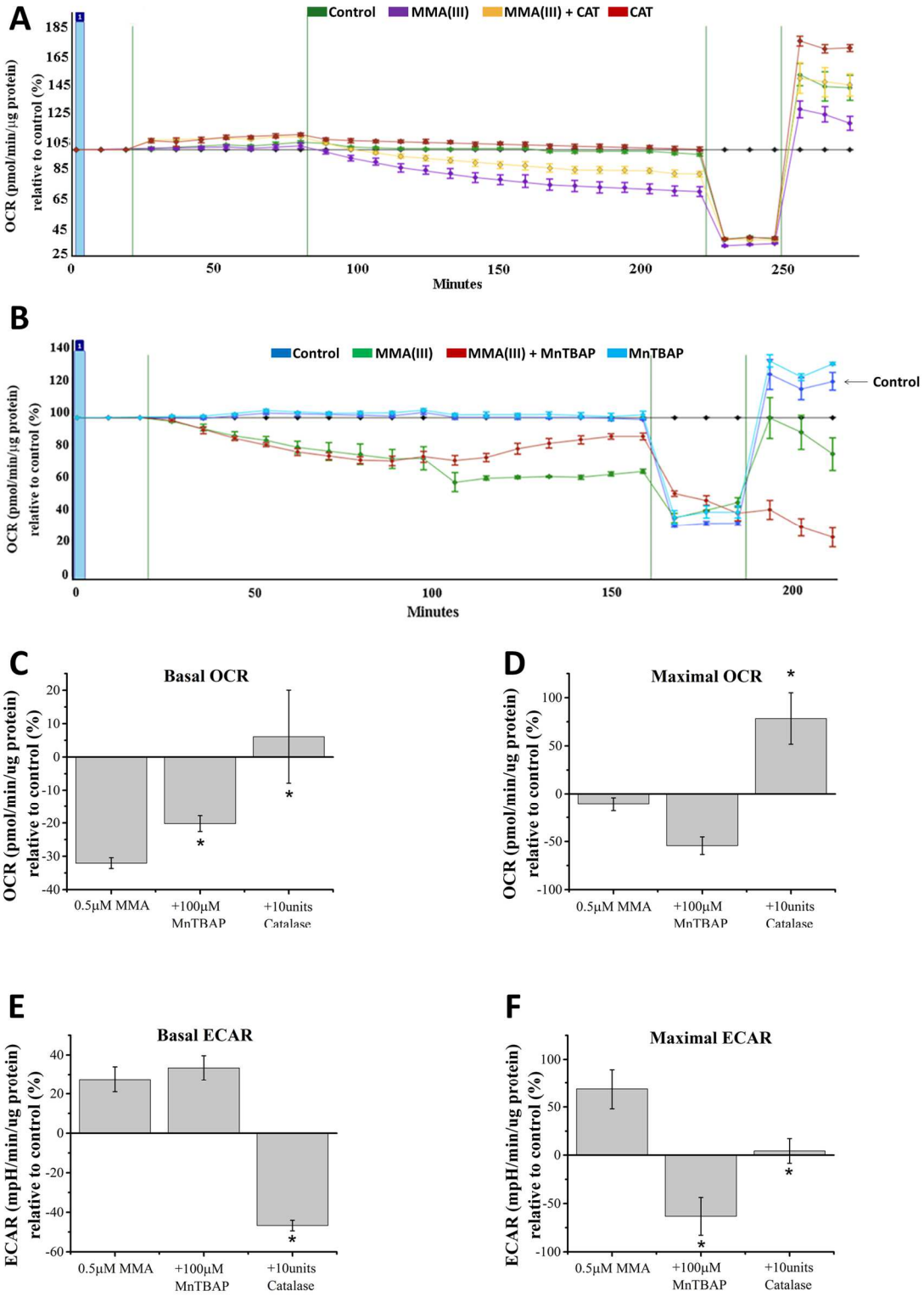
A
Control
MMA(III)
MMA(III) + CAT
CAT
OCR (pmol/min/µg protein) relative to control (%)
Minutes
B
Control
MMA(III)
MMA(III) + MnTBAP
MnTBAP
Control
OCR (pmol/min/µg protein) relative to control (%)
Minutes
C
Basal OCR
OCR (pmol/min/ug protein) relative to control (%)
0.5µM MMA
+100µM MnTBAP
+10units Catalase
D
Maximal OCR
OCR (pmol/min/ug protein) relative to control (%)
0.5µM MMA
+100µM MnTBAP
+10units Catalase
E
Basal ECAR
ECAR (mpH/min/ug protein) relative to control (%)
0.5µM MMA
+100µM MnTBAP
+10units Catalase
F
Maximal ECAR
ECAR (mpH/min/ug protein) relative to control (%)
0.5µM MMA
+100µM MnTBAP
+10units Catalase

Figure 6. Antioxidant rescue of MMA(III)-induced deficits in mitochondrial respiration.

A. Representative mitochondrial respiratory profile (oxygraph) demonstrates that co-treating cells with catalase rescued cells against 0.5μM MMA(III)-mediated decreases in baseline OCRs and maximal respiratory capacity. The oxygraph is representative of one of three experiments showing similar results.

B. Representative mitochondrial respiratory profile (oxygraph) demonstrates that pre-treating cells with MnTBAP rescued cells against 0.5μM MMA(III)-mediated decreases in baseline OCRs but not the maximal respiratory capacity. The oxygraph is representative of one of three experiments showing similar results.

C. Graph showing average baseline OCRs compiled from three experiments in cells treated with MMA(III) and co-treated with catalase or pretreated with MnTBAP for 24 hours at the indicated concentrations for 3 hours. (*:$p<0.05$ vs. 0.5μM MMA(III), Means, ±SE, n=25-29 wells/condition). One-way ANOVA, Tukey's HSD.

D. Graph showing average maximal OCRs compiled from three experiments in cells treated with MMA(III) and co-treated with catalase or pretreated with MnTBAP for 24 hours at the indicated concentrations for 3hours. (*:$p<0.05$ vs. 0.5μM MMA(III), Means, ±SE, n=25-29 wells/condition). One-way ANOVA, Tukey's HSD.

E. Graph showing average basal ECARs compiled from three experiments in cells treated with MMA(III) and co-treated with catalase or pretreated with MnTBAP for 24 hours at the indicated concentrations for 3hours. (*:$p<0.05$ vs. 0.5μM MMA(III), Means, ±SE, n=25-29 wells/condition). One-way ANOVA, Tukey's HSD.

F. Graph showing average maximal ECARs compiled from three experiments in cells treated with MMA(III) and co-treated with catalase or pretreated with MnTBAP for 24 hours at the indicated concentrations for 3 hours. (*:$p<0.05$ vs. 0.5μM MMA(III), Means, ±SE, n=25-29 wells/condition). One-way ANOVA, Tukey's HSD.

Finally, we investigated whether the reversal in mitochondrial dysfunction mediated by anti-oxidants was associated with increased cell survival against MMA(III) and iAs(III)-mediated toxicity. As proof of principle of the ROS quenching effects of antioxidants in cells are cytoprotective, we observed that treating cells with catalase or MnTBAP had a significant effect for

enhancing cell survival against both MMA(III) and iAs(III) whereas MnTBAP had a partial protective effect against MMA(III) highlighting the differential contribution of different sources of ROS on cytopathology and the ability of antioxidants to confer protection (**Figure 8**).

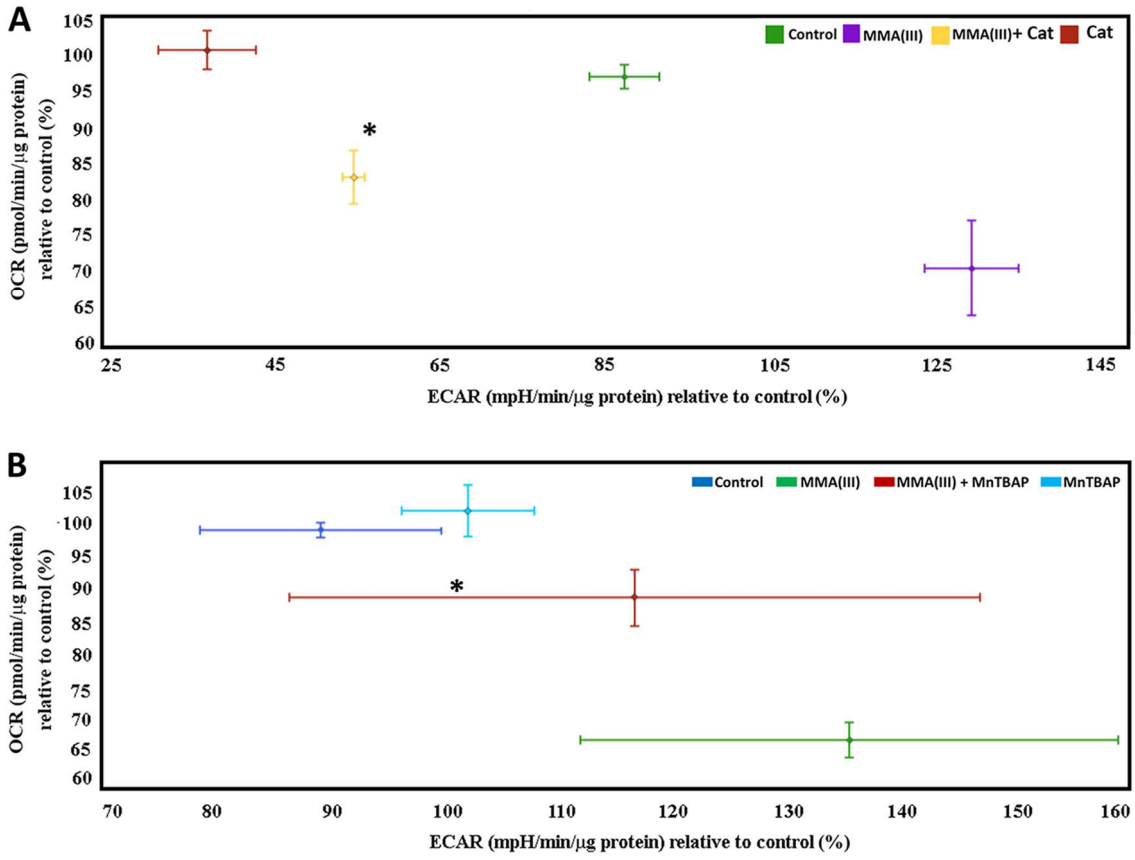

Figure 7. Antioxidants reverse the metabolic alterations induced by MMA(III).

A. Representative phenogram from one of three experiments (baseline OCRs vs. baseline ECARs) showing a robust glycolytic shift in cells treated with 0.5µM MMA(III) for 200 min. compared to control (untreated) but reversed by co-treatment with catalase (*:$p<0.05$ vs. 0.5µM MMA(III), Means, ±SE, n= 4-6 wells per condition). One-way ANOVA, Tukey's HSD. The decreased OCR observed in control group relative to its first time point likely reflects a modest induction of oxidative stress in cells incubating for 3 hours required to do the oxygen consumption assay.

B. Representative phenogram from three experiments showing (baseline OCRs vs. baseline ECARs) a robust glycolytic shift in cells treated with 0.5µM MMA(III) compared to control

(untreated). MnTBAP pre-treatment was only able to partially reverse the glycolytic shift (*:p<0.05 vs. 0.5μM MMA(III), Means, ±SE, n= 4-6 wells per condition). One-way ANOVA, Fishers LSD. The decreased OCR observed in control group relative to its first time point likely reflects a modest effect oxidative stress in cells incubating for 3 hours required to do the oxygen consumption assay.

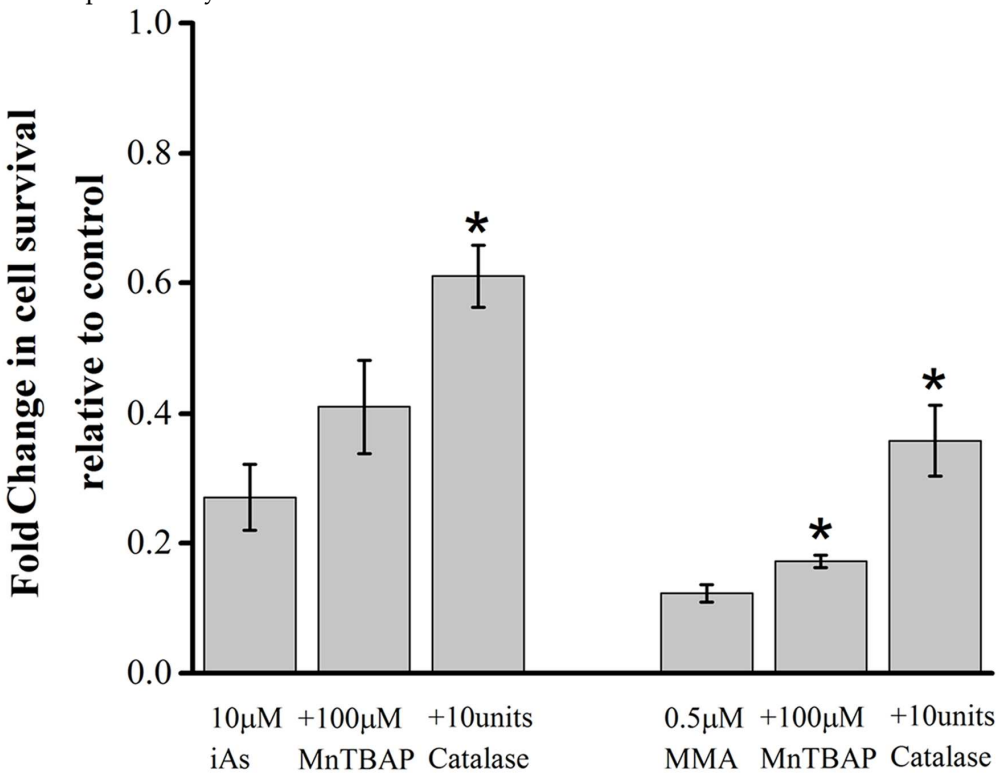

Figure 8. Antioxidants reverse cell death induced by MMA(III) and iAs(III).

Compiled quantification of cell viability as measured by the MTS assay in cells treated with the indicated concentrations of MMA or iAs(III), and either pretreated with MnTBAP or co-treated with catalase. (*:p<0.05 vs. 10μM iAs(III) or 0.5μM MMA(III) of their respective groups, **:p<.05 comparing MnTBAP to catalase treatment in respective groups, Means, ±SE, n=6-21 wells/condition). One-way ANOVA, Tukey's HSD. Data was compiled from three experiments.

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vi. Discussion

Although it is widely accepted that arsenic species can produce various degrees of mitochondrial dysfunction in myriad cell types, the effects of inorganic and organic species of arsenic on mitochondrial function and structure

in VSMCs have not been elucidated. In this study, we demonstrate for the first time that MMA(III) promotes mitochondrial dysfunction and oxidative stress in VSMCs, and that these deficits stem from the generation of mitochondrial and non-mitochondrial ROS following exposure to MMA(III).

vi.i. MMA(III) induces cytotoxicity in VSMCs

Our findings on the cytotoxicity of arsenic to VSMCs are in agreement with a majority of studies demonstrating arsenic's capacity to induce apoptosis in various cell lines including aortic, coronary, and mesenteric vascular smooth muscle [139,255,261-263]. Our data shows that MMA(III), but not iAs(III), promotes caspase-dependent apoptosis in A7r5 cells consistent with other studies [138-140], whereas iAs(III) predominantly elicits necrosis [140] (**Figure 1, S4**). Furthermore, several studies suggest that apoptosis of smooth muscle cells may play a pathological role in cardiovascular disease progression [256,282,283], suggesting that cell death of vascular smooth muscle cells may underlie pathology in CVDs associated with arsenic intoxication.

vi.ii. Arsenic targets mitochondria

Organic species of arsenic demonstrate higher toxicity compared to iAs in a variety of cell types. For instance, MMA(III) can induce apoptosis in the human intestinal epithelial cell line Caco-2 by elevating ROS, inducing a reduction in intracellular GSH, and increasing peroxidation of lipids. MMA(III) also alters the antioxidant activities of glutathione peroxidase and catalase and concomitantly induces expression of heat shock proteins and metallothioneins [139]. There is a wealth of evidence that supports selective toxicity of arsenic species towards mitochondria. Previous studies have shown that arsenic induces widespread mitochondrial dysfunction via loss of mitochondrial membrane potential, generation of ROS, diminution of cytochrome c oxidase function, and suppression of oxygen consumption via depletion of mtDNA copy number [143,144]. Consistent with the toxic effects of MMA(III) on mitochondria in many cell types, our data also show that MMA(III) is a mitochondria-specific toxicant in VSMCs. Our study is the first to show an interplay and temporal dynamics of MMA(III) exposure, generation of mitochondrial superoxide and non-mitochondrial ROS, and alterations in mitochondrial function and metabolism in VSMCs (**Figure 2A-C, Figure 4**).

Our mitochondrial functional assays suggest that MMA(III) is a stronger inducer of mitochondrial dysfunction upstream of cell death compared to iAs(III) or to MMA(V) at equimolar concentrations that are sufficient to induce significant mitochondrial superoxide levels but not high enough to induce cytotoxicity (**Figure 2, Figure S4**). Although some doses of arsenicals used in this study may be considered high, humans have been acutely and chronically exposed to similar concentrations of arsenicals environmentally [176] and in the clinical treatment of acute promyelocytic leukemia [177]. Furthermore many epidemiological studies have demonstrated the pathological effects of long-term accumulation of arsenicals in human bodies at high doses [43,50]. Nevertheless, our study raises the possibility that mitochondrial dysfunction/metabolic alterations induced by MMA(III), at the prelethal doses used, may contribute to pathology and metabolic alterations in humans exposed to arsenic. Indeed, MMA(III)-induced perturbations of glycolytic flux, as discussed in the following section, are particularly relevant to vascular smooth muscle function due to the observation that ATP generated from glycolysis may have a separate 'metabolic purpose' (e.g., provision of energy for the Na^+/K^+ antiporter) than ATP derived from oxidative phosphorylation, which has been demonstrated to provide energy to primarily support contractile functions [284]. MMA(III)-induced shifts

in ATP generation may therefore have profound consequences for VSMC function, especially since these cells are reliant on phosphocreatine (PCr) for short-term, instantaneous metabolic demands and PCr is resynthesized mainly via oxidative phosphorylation [285]. Overall, our data strongly suggest that MMA(III) is a highly selective arsenic metabolite that targets mitochondrial function at sublethal concentrations and time points. However, we do not rule out the possibility that iAs(III) may also contribute to mitochondrial pathology, albeit less efficiently than MMA(III), in VSMCs in instances of prolonged exposure to iAs(III).

vi.iii. Selective toxicity of MMA(III) to mitochondria activates metabolic compensation in VSMCs

Our results show that treatment of VSMCs with MMA(III) led to significant decreases in basal and maximal OCRs while concomitantly enhancing basal and maximal ECARs compared to cells treated with iAs(III) (**Figure 2**). The ECAR values detected by the XF24e analyzer are a reliable measure of glycolytic rate. Hence, a decrease in the ratio of the mean OCR to ECAR associated with MMA(III) exposure (**Figure 7**) is an indicator of a compensatory upregulation of glycolysis as a result of mitochondrial dysfunction. Given that MMA(III) is

known to adversely affect the functioning of the electron transport chain (ETC) [250], an increase in glycolysis may be a compensatory mechanism to ensure cellular survival, somewhat akin to the Warburg effect [276] due to mitochondrial impairment. However, it is worth noting that this increase in ECARs caused by MMA(III) exposure does not lead to an increase in cytosolic ATP levels under conditions that favor mitochondrial ATP production (**Figure 3**), or in glycolytic conditions (data not shown), suggesting that either MMA(III) elicits a futile upregulation of glycolysis or that non-mitochondrial sources of ROS (e.g. hydrogen peroxide from ER) shut down compensatory glycolysis as suggested by our ATP assays that show a significant decrease in cytosolic ATP at 1μM MMA(III). Toxic stimuli that elicit classical apoptosis (e.g. staurosporine) can significantly elevate cytosolic ATP levels to promote cell death [286]. Although MMA(III) elicits apoptosis as evident by increased active caspase-3 levels and nuclear fragmentation (**Figure S4 A-B**), our luminescence-based ATP assays show that MMA(III) does not elicit an increase, but modestly decreases, cytosolic steady-state ATP levels at 2 or 6 hour of exposure (data not shown). This observation suggests that MMA(III) may promote apoptosis through distinct mechanisms that do not involve a transient rise in cytosolic ATP levels compared to "classical" apoptosis induced by staurosporine, or that non-

mitochondrial ROS may prevent an upregulation of cytosolic ATP levels by inactivating glycolytic enzymes.

Our data also suggests that the decline in mitochondrial respiration resulting from MMA(III) is an early event that occurs in response to elevated mitochondrial superoxide levels and precedes the loss of mitochondrial ATP and content (**Figures 2-5**). Although iAs(III) toxicity does not lead to early mitochondrial dysfunction, prolonged exposure of VSMCs with iAs(III) can lead to a reduction in mitochondrial content, fragment mitochondria, and elevate mitochondrial superoxide levels suggesting that iAs(III) induces delayed mitochondrial toxicity without inducing significant cell death (**Figure, S4, S7-9**).

vi.iv. What are the mechanisms by which arsenic decreases mitochondrial content in VSMCs?

We observed that treating VSMCs with MMA(III) was associated with robust aberrant perinuclear aggregation (clustering) of mitochondria in a subpopulation of cells (**Figure 5F**) and significant loss of mitochondria (**Figure 5B-E**). The effects of MMA(III) on mitochondrial aggregation (clumping or perinuclear clustering) is consistent with another study which reported mitochondrial aggregation in the arsenic trioxide -sensitive A172 human

glioblastoma cells [287]. In yet another study, chronic treatment of AL human-hamster hybrid CHOK1 cells with arsenic resulted in an abnormal distribution of mitochondria that varied considerably between cells including increased mitochondrial fusion or filamentous morphology. This effect was associated with decreased mitochondrial function [144].

In mammalian cells, the E3 ubiquitin ligase Parkin is known to promote perinuclear clustering of damaged mitochondria to facilitate mitophagy [288]. Indeed, Watanabe et al., (2014) recently reported that ubiquitin proteasome system activity is increased by arsenic trioxide and the autophagy lysosomal system is increased in mouse atrial cardiomyocytes suggesting that the elimination of dysfunctional mitochondria is enhanced by the E3 ubiquitin ligase Parkin in arsenic-treated cells [289]. Based on our morphological analyses of perinuclear clustering and mitochondrial loss, it is conceivable that the process of elimination of mitochondria induced by both MMA(III) and iAs(III) may involve Parkin-mediated mitophagy. Hence, our study warrants future investigations to elucidate mechanisms of mitochondrial elimination by MMA(III) in VSMCs.

vi.v. Contribution of different sources of ROS induced by arsenic

There is significant evidence in the scientific literature indicating that arsenic exposure results in increased production of ROS in different cell types including Neuro-2a cells, β- pancreatic cells, human hepatocellular carcinoma HepG2 cells, and human breast and lung carcinoma cell lines [290,291]. However, little is known regarding the subcellular sources of ROS induced by arsenic compounds. In our study, MMA(III) induced a significant elevation of mitochondrial superoxide and hydrogen peroxide levels (**Figure 4A-B**). At 4 hours post exposure to MMA(III), the levels of superoxide generated are similar to levels achieved in cells treated with the complex I inhibitor rotenone. iAs(III) treatment also induces a modest increase in mitochondrial superoxide production at 4 and 6 hours post treatment suggesting that MMA(III) is a more potent inducer of mitochondrial ROS in VSMCs.

Although it is clear that mitochondrial superoxide induced by MMA(III) contributes to mitochondrial dysfunction in VSMCs (**Figure 2 and 3**), our data suggest that non-mitochondrial sources of hydrogen peroxide also contribute to mitochondrial dysfunction (**Figure 8**). While pretreating VSMCs with catalase was sufficient to reverse MMA(III)-mediated decreases in basal and maximal OCRs and block the increase in ECARs (**Figure 6C-D**), MnTBAP was incapable of

rescuing all aspects of mitochondrial function (e.g. maximal respiration) (**Figure 6B and D**). Unexpectedly, although MnTBAP pretreatment partially rescued basal OCRs, it worsened the reduction in maximal OCRs induced by MMA(III) treatment (**Figure 6D**). Thus, treating VSMCs with catalase, but not MnTBAP, reversed the energy profile from a predominantly glycolytic to normal phenotype as untreated cells (**Figure 6A**).

It is worth noting that mitochondria may be the primary target of different arsenic compounds, but are not the sole contributors to ROS production. Indeed, the endoplasmic reticulum and peroxisomes have a greater capacity to produce ROS than mitochondria in liver tissue [292]. Additionally, arsenic has been shown to induce ROS by deregulating NADH/NADPH oxidase activity in VSMCs and endothelial cells, leading to an increase in superoxide and hydrogen peroxide levels [246], and arsenic-induced ROS couples with nitric oxide (NO) to produce peroxynitrite [253]. Thus, in addition to mitochondrially derived ROS, non-mitochondrial ROS induced by iAs(III) and MMA(III) likely elicit oxidative damage in other organelles in our experimental model. Hence, our work warrants future studies that identify all mitochondrial sources of ROS, and verify non-mitochondrial sources of ROS induced by MMA(III) that contribute to cellular pathology in VSMCs through previously elucidated pathways [246,253].

A major limitation of the present study is the use of an immortal cell line for linking pathological effects of arsenic toxicity with chronic effects of arsenic toxicity as related to toxicity in humans. However, the benefits of using the immortalized A7r5 cell line as an *in vitro* model of arsenic toxicity in smooth muscle cells are to unveil mechanisms of arsenic toxicity in VSMCs using an acute dosing paradigm include robust consistency, reproducibility of results, a high availability of biomass to do *in vitro* studies *in lieu* of primary cells which precludes the need to sacrifice animals, and a lack of phenotypic change due to sub-selection in passaging. Thus, given the mitochondrial-toxic effects of MMA(III) as shown in this report, our studies warrant future experiments that analyze the toxic effects of MMA(III) vs. iAs(III) in a chronic cell culture model of arsenic exposure to elucidate the role that MMA(III) plays in VSMC toxicity.

vii. Conclusion

In summary, our study is the first to show that MMA(III) can induce concomitant mitochondrial dysfunction, mitochondrial ROS and metabolic alterations in VSMCs. Our study suggests that MMA(III) elicits mitochondrial and non-mitochondrial ROS to promote early mitochondrial dysfunction by affecting oxidative phosphorylation, ATP content, and mitochondrial

morphology resulting in a pathology that precedes the loss of mitochondrial content. Our work warrants future studies that probe for molecular mechanisms by which MMA(III) selectively targets mitochondria and elicits the release of superoxide in VSMCs.

viii. Conflict of interest statement

The authors have no conflict of interests to declare for this study.

ix. Acknowledgements

We would like to acknowledge Dr. Carlos Martino for his technical support and assistance in optimizing the Amplex Red assays. We would like to also acknowledge Dr. Stephen Spain and Casey Philbin of the UNR Chemistry Departments' Shared Instrumentation Laboratory (SIL) for their assistance with assessment of the purity and synthesis of MMA(III) stocks by ^{1}H-NMR. This research was funded in part by NIH/NIGMS #GM103554 (a COBRE grant in "Cell Signaling Across Membranes"), and by start-up funds from the University of Nevada School of Community Health Sciences and the Department of Pharmacology of the University of Nevada School of Medicine.

Supplemental Figures.

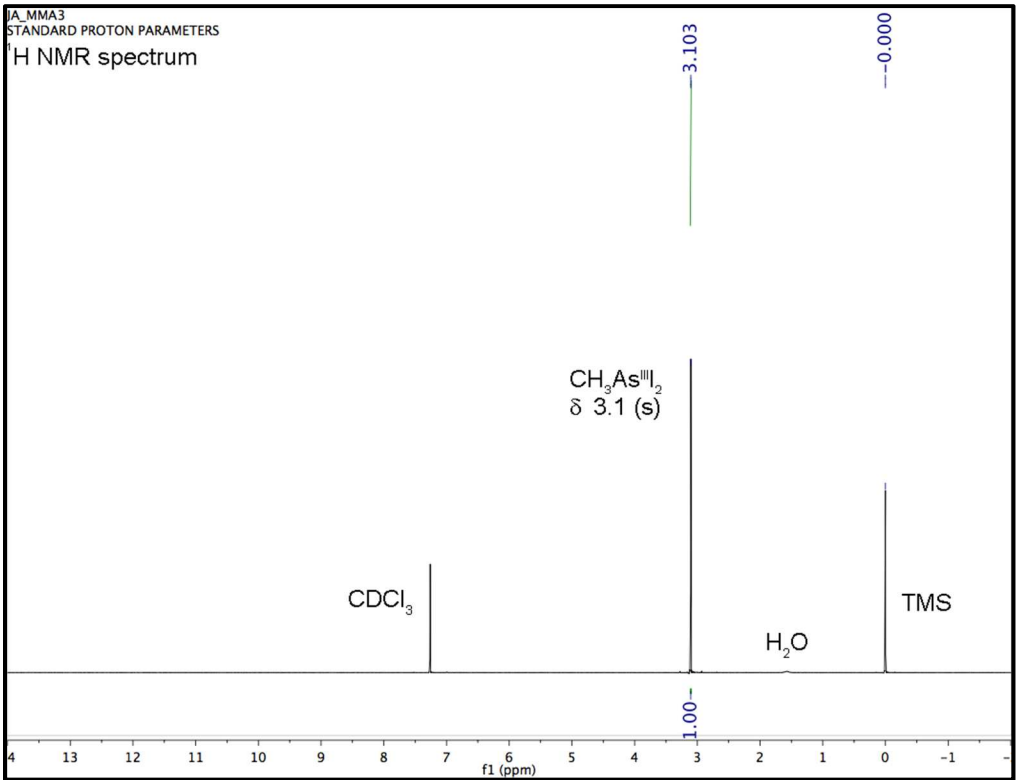

Figure S1. Identity and purity of MMA(III) as verified by ^{1}H-NMR.
5.8mg $CH_3As^{III}I_2$ was dissolved in 0.7mL ($CDCl_3$ + 0.05% TMS) and the ^{1}H-NMR experiment was conducted on a Varian 400-MR liquid state NMR. The NMR spectrum indicates the expected shift for $CH_3As^{III}I_2$ at ⌾=3.1 ppm (Styblo *et al*, 1997) and the absence of a proton shift at ⌾=2.0 ppm, demonstrating the absence of any contaminating dimethylated product (DMA(III)). TMS: tetramethylsilane, an internal standard. $CDCl_3$: deuterated chloroform, a solvent used to dissolve $CH_3As^{III}I_2$

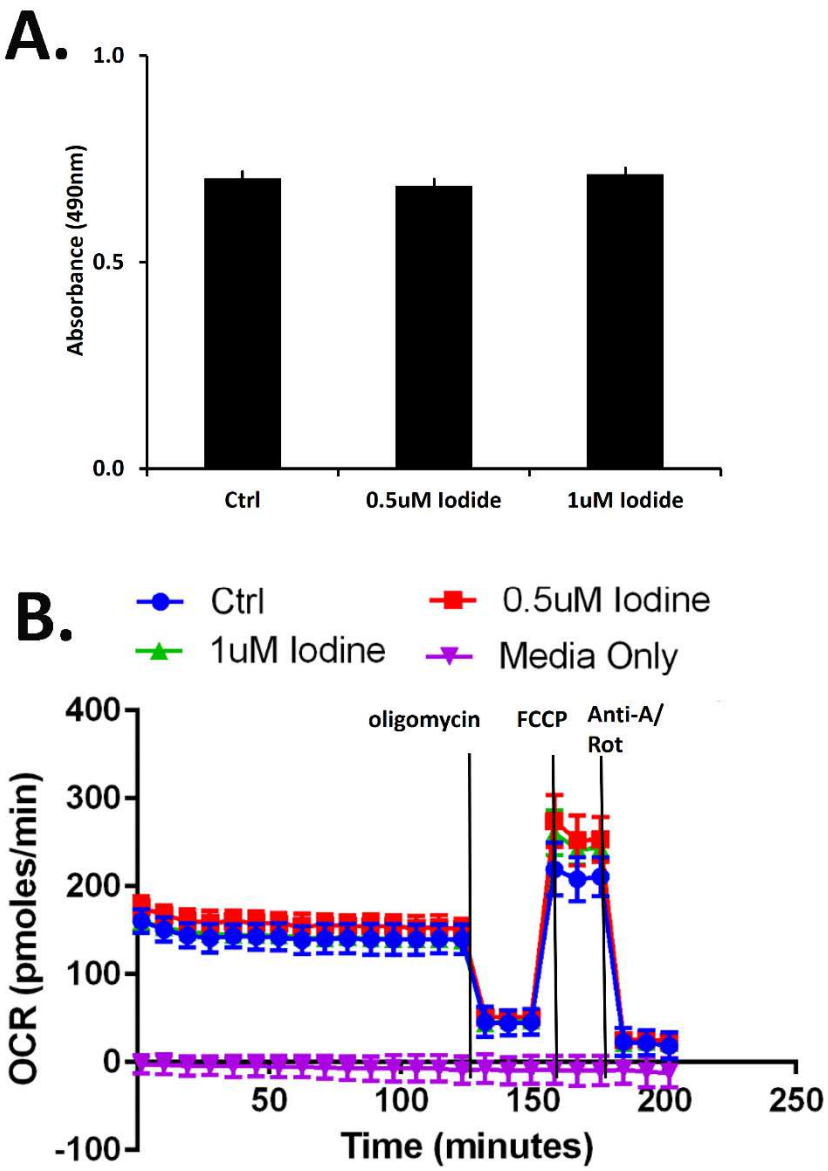

Figure S2. Effects of iodide on cell viability and mitochondrial respiration. **A)** A7r5 cells seeded in 96 well plates were treated with increasing concentrations of iodide as found in 0.5μM or 1μM MMA(III) or with water alone (control, Ctrl) for 24 h. Cell viability was assessed by using the MTS assay. The bar graph shows that increasing concentrations of iodide does not significantly affect cell viability. Means ± SD, n=8 wells per condition). No significant differences found by One-Way ANOVA for any time points, followed by Fisher's LSD. **B)** Representative OCR trace of A7r5 cells treated with either vehicle control (water) or with increasing concentrations of iodide for two h. prior to being sequentially exposed to oligomycin, FCCP and antimycin-A/rotenone to measure baseline, ATP-linked, maximal, and non-mitochondrial respiration respectively. The OCR trace shows that iodide does not significantly affect mitochondrial respiration.

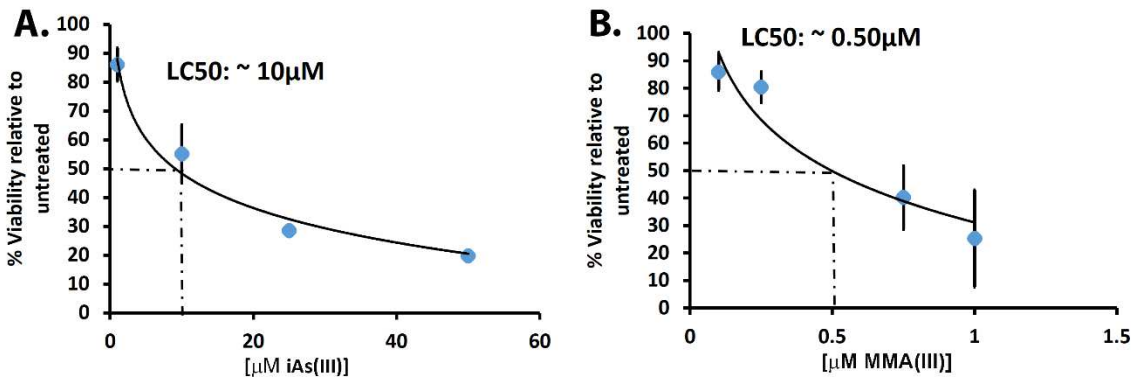

Figure S3. Cell density counts verify the 24hour- LC50s observed by MTS and Trypan Blue assays. Quantification of cell viability of A7r5 cells treated with increasing concentrations of either iAs(III) (**A**) or of MMA(III) (**B**) as assessed by performing cell counts with a Coulter counter. The mean cell count per dose of arsenical was plotted as the logarithmic line graph and the LC50s at 24 h. (0.5µM for MMA(III) and 10µM for iAs(III)) are indicated by dashed lines. Means ±SD, n=4 wells. Each line graph is representative for one of two experiments with similar results.

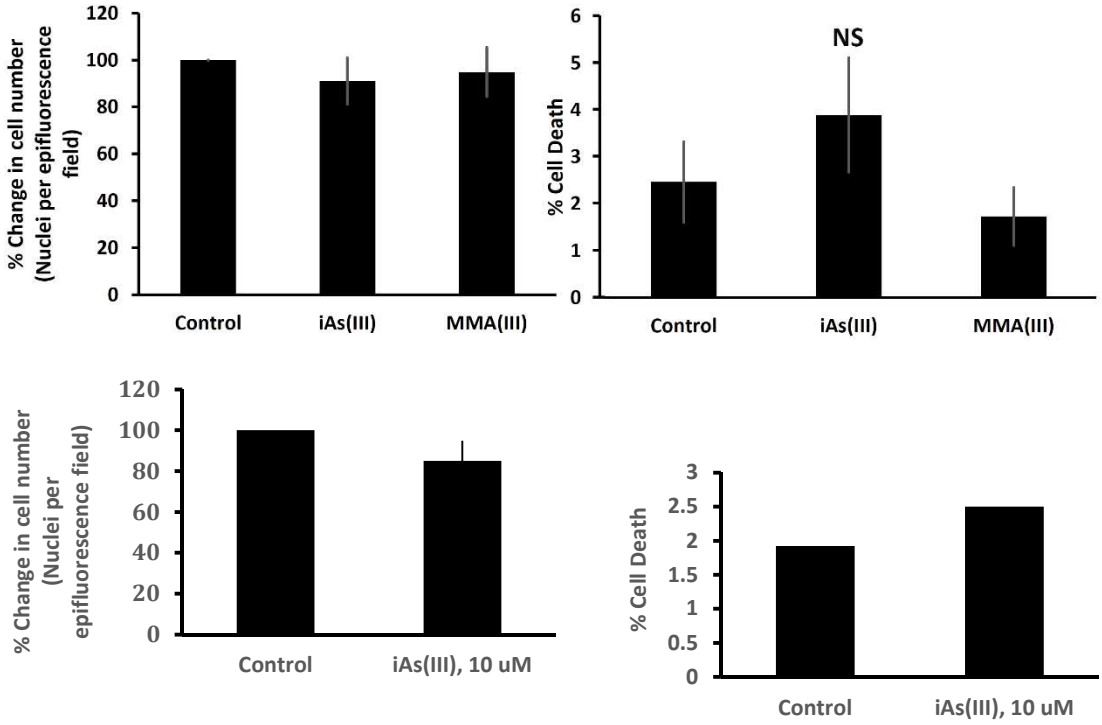

Figure S4. A 6 hour treatment of cells with 0.5μM or 1μM MMA(III) and 1μM or 10μM iAs(III) does not significantly promote cell death.

A7r5 cells were treated with 1μM concentrations of MMA(III) or iAs(III) (Upper left graph) or 10μM iAs (Lower left graph) for 6 h. prior to fixation with paraformaldehyde and counterstaining with DAPI to visualize nuclei. The average number of DAPI-stained nuclei per epifluorescence micrograph was counted as an index of cell density. The percentage of cells containing fragmented or pyknotic nuclei per epifluorescence micrograph was counted as an index of active cell death. The data shows that a 5 hour treatment of cells with arsenicals does not significantly induce cell death or reduce cell number ruling out that the pathological/cytotoxic effects of MMA(III) on mitochondrial content and morphology (**Figure 5**) is a result of cytotoxicity. (**NS**: not significant vs. untreated, Means, ±SD, n=500-600 cells/condition). One-way ANOVA, Tukey's HSD. Data was compiled from three independent experiments for 1μM concentrations, and data is representative of one experiment for 10μM iAs.

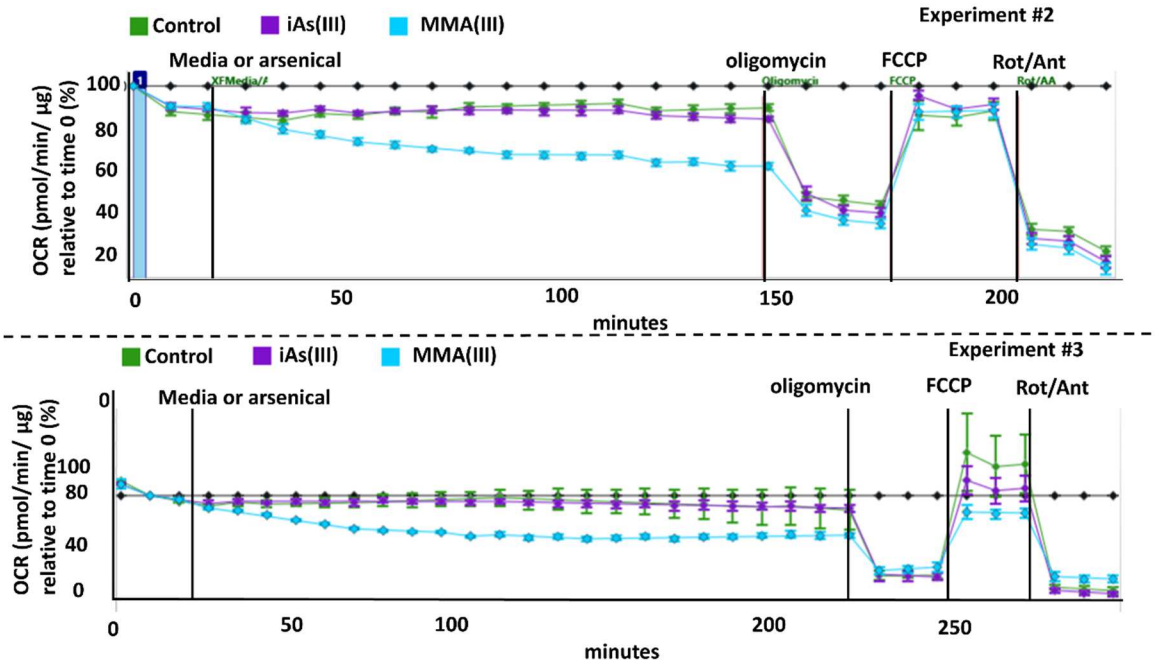

Figure S5. Other representative oxygraph traces showing that MMA(III) but not iAs(III) reduces OCRs in A7r5 cells.

Representative mitochondrial respiration profiles (oxygraph) of two other representative experiments showing that 0.5 µM MMA (III) (top profile), but not 10µM iAs(III) (bottom profile) progressively decreases OCRs and this effect plateaus by 100 minutes of treatment. In addition, there is a decrease in ATP-linked mitochondrial respiration as well.

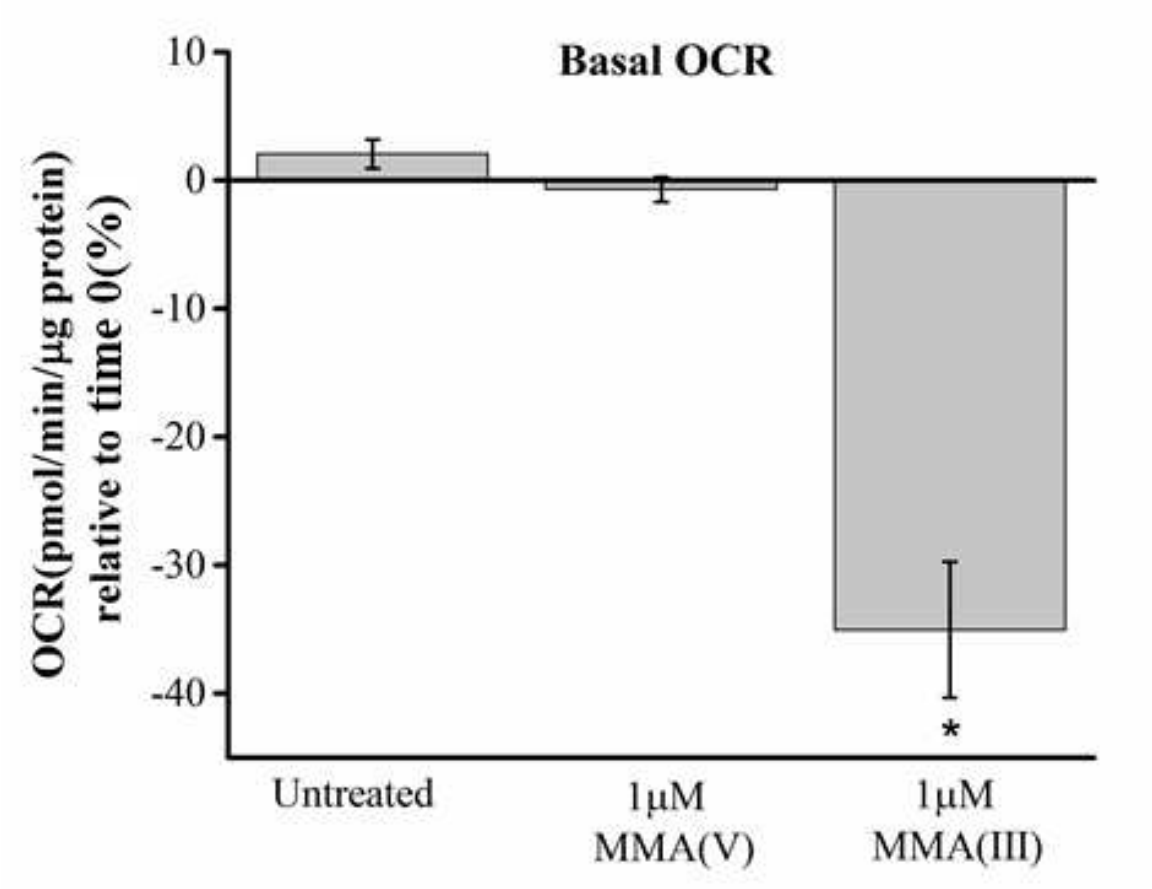

Figure S6. MMA(III) but not MMA(V) decreases the OCRs in A7r5 cells.
Quantification of the average OCRs in A7r5 cells treated with a 2 h. dose of the indicated compounds. A decrease in baseline mitochondrial respiration was observed in cells treated with MMA(III) but not MMA(V), (*:p<.05 vs. 1μM MMA(V)), Means ± SD, n=3-4 wells per condition). One-way ANOVA, Tukey's HSD. Data is representative for one of two experiments with similar results.

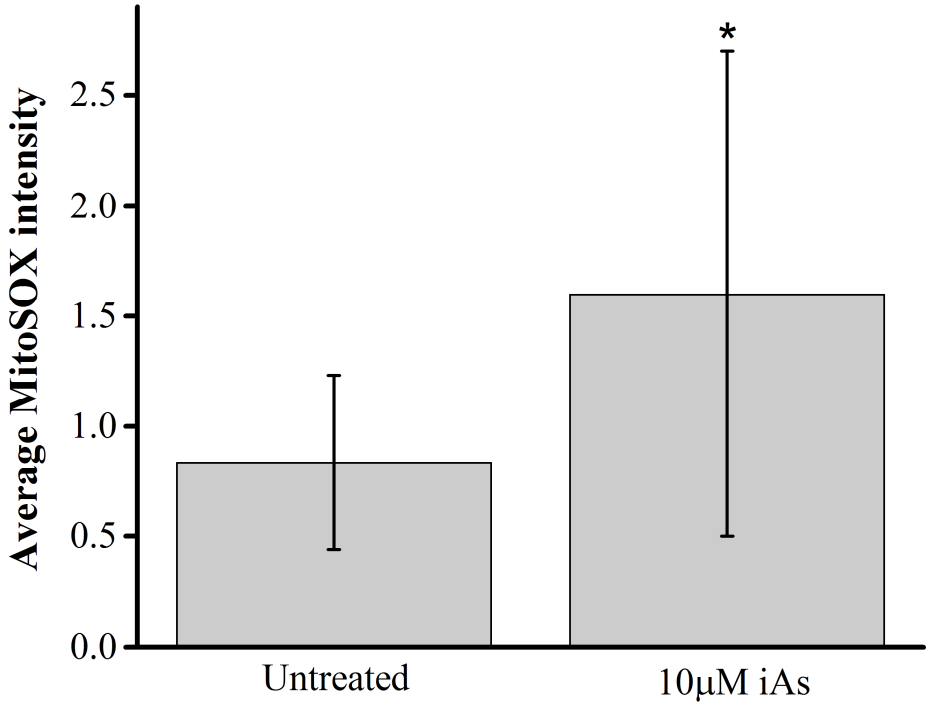

Figure S7. iAs(III) can induce a significant increase in mitochondrial superoxide levels in A7r5 cells at 6 hours of treatment. Quantification of the average intensity of MitoSOX fluorescence in untreated cells or in cells exposed for 6 h. with 10μM iAs. A significant elevation in MitoSOX intensity was detected among cells treated for 6 h. with 10μM iAs, (*:p<.05 vs. untreated, Means ± SD, n=26 cells/condition). Independent t-test. Data is representative for one experiment.

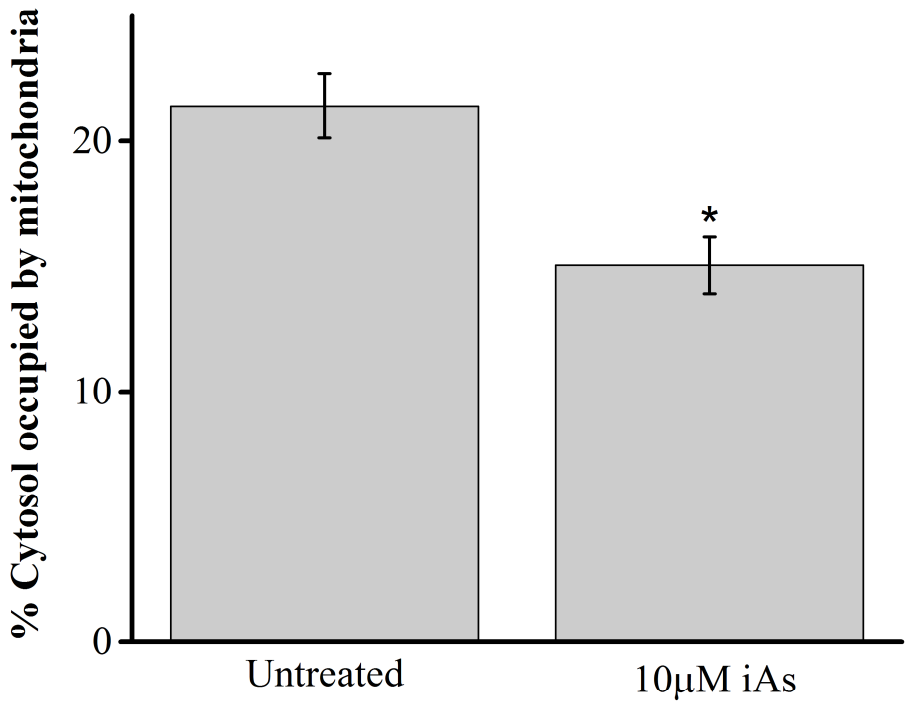

Figure S8 iAs treatment induces a decrease in mitochondrial content in A7r5 cells at 5 h of treatment. Imaged-based quantification of mitochondrial content as determined by measuring the percentage of the cytosolic area occupied by fluorescently labeled mitochondrial specific pixels in cells treated for 5 h. with iAs and immunolabeled for mitochondrial marker TOM20. A significant decrease in mitochondrial content was detected among cells exposed to 10μM iAs(III) vs. untreated cells (*:$p<0.05$ vs. untreated, Means, ±SD, n=26 cells/condition). Independent samples t-test. Data is representative of one experiment.

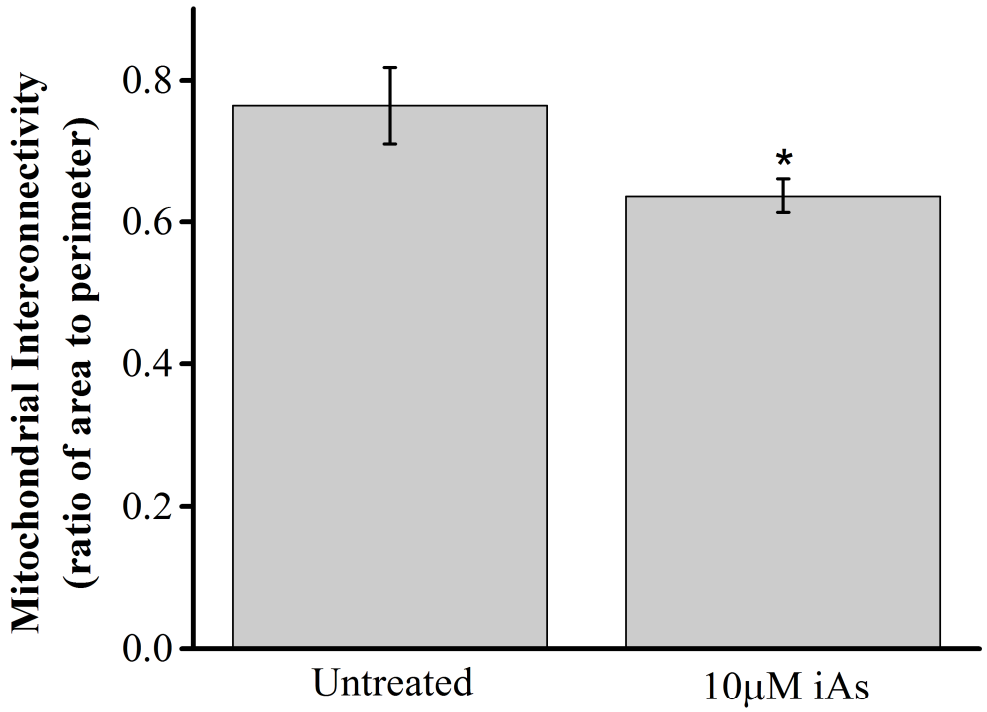

Figure S9. iAs fragments mitochondria in A7r5 cells at 6 hours of treatment.
Mitochondrial interconnectivity, defined as the ratio of the average area to the perimeter of mitochondria per cell (Dagda et al. 2009), experienced a significant decrease among cells treated for 6 h. with an LC50 of iAs(III) vs. untreated cells (*:$p<0.05$, Means, ±SD, n=27-28 cells/condition). Data is representative of one experiment.

Chapter 5. Summary, conclusions and recommendations

This dissertation, methodologically structured as a review of published data, a secondary data analysis, and an original study of experimental data, contributes to the literature on arsenic and cardiovascular disease. Chapter 2 identifies 22 compounds with antioxidant properties that are effective against arsenic induced mito-toxic effects in cardiovascular tissue and cells. These data suggest that antioxidants have the potential to improve the cardiovascular health of millions of people chronically exposed to elevated arsenic concentrations through contaminated water supplies or lifesaving cancer treatment. We recommend future studies comparing antioxidant ability to prevent arsenic induced mito-toxic effects in H9c3 cardiomyocytes and vascular smooth muscle cells.

Additionally, we recommend developing mitochondrially targeted nanoformulation for improved antioxidant pharmacokinetics for use *in vivo* and *in vitro*. Although substantial preliminary research is necessary, we suggest that antioxidants have a potential for pharmaceutical development into products to counteract arsenic-induced cardiovascular toxicity in a real-world context.

Chapter 3 is the first study to examine urine arsenic methylation with respect to metabolic disorder in the U.S. We report a significant association

between arsenic methylation capacity and metabolic syndrome women with normal BMI in a country where inorganic arsenic is regulated at 10 ppb. This suggests that current MCL should be evaluated in terms of cardiovascular outcomes using a model that establishes causality.

In chapter 4 we demonstrate increased toxicity of the arsenic metabolite MMA(III) relative to iAs(III) in VSMCs. We report that MMA(III) promotes mitochondrial dysfunction and oxidative stress in VSMCs, and that these deficits stem from the generation of mitochondrial and non-mitochondrial ROS following exposure to MMA(III). This work warrants future studies that probe for molecular mechanisms by which MMA(III) selectively targets mitochondria and elicits the release of superoxide in VSMCs.

Increased toxicity of methylated arsenic species (demonstrated in chapter 4) support the outcome that increased arsenic methylation is associated with increased odds of metabolic syndrome (demonstrated in chapter 3), this may be due to the elevated presence of toxic methylated species that result from increased arsenic methylation. These data supports the concept that increased arsenic methylation increases the concentrations of toxic methylated arsenic species in the body, resulting in increased rates of ROS production, and contributing to disease status. Our results from chapter 2 (antioxidant

amelioration of arsenic-induced cardiovascular dysfunction) provide suggestions for research on the prevention of arsenic-induced cardiovascular dysfunction.

It is worth noting that in chapter 4 we found that MMA(III) induced apoptosis, while iAs(III) primarily induced necrosis [57]. This finding is supported by a variety of other studies, however, our literature review (chapter 2) includes reports that iAs(III) induces both apoptosis and necrosis. In addition, in chapter 4, we report that in a study conducted by Naranmandura et al. (2011) MMA(III), but not iAs(III) selectively targeted and inhibited mitochondrial complexes II and IV in isolated mitochondria from rat liver [250], however, chapter 2 included two studies that reported a decreased activity at mitochondrial complexes I, III, and IV that was induced with iAs(III) [122] and 2 studies that reported decreased activity at mitochondrial complex II that was induced with iAs(III) [122]. We believe these are examples of the varying effects of arsenic that depend on dose and duration of exposure as well as cell/tissue type. These apparent contradictions highlight the need for additional study, given that the effects of arsenic are often non-linear.

In light of evidence that trivalent arsenic species are selectively toxic to mitochondria, and that arsenic biotransformation is mediated by methylation capacity, we recommend additional studies that consider the contribution of

genetic polymorphisms on arsenic metabolism in relation to pre- and post-clinical cardiovascular disease status.

Improved knowledge of genetic polymorphisms that contribute to methylation capacity, namely, AS3MT and GST, could play a critical role in understanding the relationship between arsenic toxicity, oxidative stress, and cardiovascular disease /metabolic syndrome. Therefore, we suggest that future studies investigate the role of arsenic exposure and genetic polymorphisms that contribute to arsenic methylation capacity on cardiovascular disease outcomes using a model that establishes causality.

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