Sex and diet as modulators of arsenic-induced atherosclerosis in apolipoprotein E knockout mice

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Dedication

I dedicate this thesis to all the countless laboratory animals

involved in this project and throughout the history of scientific exploration.

As humans, we should be eternally grateful for their ultimate sacrifice.

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List of Abbreviations

WHO	World health organization
ABCA1	ATP-binding cassette transporter member 1
ABCC1	ATP binding cassette sub family C member 1
ABCC2	ATP binding cassette sub family C member 2
ABCC4	ATP binding cassette sub family C member 4
AHA	American heart association
ALT	Alanine transaminase
$apoE^{-/-}$	Apolipoprotein E knockout
AQP3	Aquaporin 3
AQP7	Aquaporin 7
AQP9	Aquaporin 9
As_2O_3	Arsenic trioxide
AS3MT	Arsenic 3 methyltransferase
As-GSH	Arsenic-glutathione
AST	Aspartate transaminase
ASTDR	Agency for Toxic Substances and Disease Registry
ATG	Arsenic triglutathione
ATP	Adenosine triphosphate
CD36	Cluster of differentiation 36
CRP	C-reactive protein
DMAG	Dimethylasrinic glutathione
DMA^{III}	Dimethylarsenous acid
DMA^{V}	Dimethylarsinic acid
GLUT1	Glucose transporter 1
GSH	Glutathione
GSTO1	Glutathione S-transferase omega-1
HDL	High-density lipoprotein
HFD	High fat diet
HMW	High molecular weight
HNE	4-hydroxy-trans-2-nonenal
ICAM-1,	Intracellular adhesion molecule 1
IL-1	Interleukin-1
$LDLR^{-/-}$	LDL receptor knockout
LDLs	Low-density lipoproteins
LOX-1	Lectin-like oxidized low density lipoprotein receptor 1
MADG	Monomethylarsonic diglutathione
MAs^{III}	Methylarsenous acid
MCP1	Monocyte chemoattractant protein 1

M-CSF	Macrophage colony-stimulating factor
MDA	Malondialdehyde
MMA^{III}	Monomethylarsonous acid
MMA^{V}	Monomethylarsonic acid
MOMA-2	Macrophages/Mononcytes monoclonal antibody 2
OMS	l'Organisation mondiale de la santé
PD	Purified diet
PIT1	Phosphate transporter 1
PIT2	Phosphate transporter 2
ppb	Parts per billion
ppm	Parts per million
ROS	Reactive oxygen species
SAM	S-adenosylmethionine
Srebp1c	Sterol regulatory element binding protein 1
Thbs1	Thrombospondin-1
TMA^{III}	Trimethyl arsine
$TMAO^{V}$	Trimethyl arsine oxide
TNF-α	Tumor necrosis factor alpha
USA	United States of America
VACM-1	Vascular cell adhesion molecule 1
α-SMA	A-smooth muscle actin

Abstract

Arsenic is identified as one of the most important chemical contaminants worldwide by the World Health Organization (WHO). Yet, millions of people are still exposed on a daily basis to arsenic through contaminated air, soil, and groundwater due to both natural and anthropogenic sources. Even though arsenic concentrations are heavily regulated in the workplace, in agriculture, and in the municipal drinking water, arsenic exposure remains a significant threat to human health. Arsenic exposure causes a myriad of adverse health effects, including neurological and skin disorders, various cancers, and cardiovascular diseases, such as atherosclerosis. Atherosclerosis is both a cardiovascular disease and an immune disease, mostly associated with the narrowing and hardening of arteries due to fibro-fatty build up involving various immune cells, collagen, and oxidized cholesterol. Due to the high cholesterol content, a high fat diet is a known contributor to atherosclerosis, however, there are sexual dimorphisms between how males and females differ in atherosclerosis development.

Previously, our lab has shown that arsenic increases the plaque size in the aortic sinus in male apolipoprotein E knockout (apoE$^{-/-}$) mice in the absence of high fat diet, while altering various plaque constituents independently from diet. While macrophage levels stayed consistent, lipid levels increased, whereas levels of collagen and smooth muscle cells decreased. However, arsenic-induced atherosclerotic plaques have never been characterized in females. Thus, in this study, we investigated the role of sex and diet in arsenic-induced atherosclerosis in apoE$^{-/-}$ female mice both in the presence and absence of high fat diet by:

1) Measuring the size of the arsenic-induced atherosclerotic plaques in the aortic sinus.
2) Measuring the levels of key constituents of the arsenic-induced atherosclerotic plaques in the aortic sinus, such as lipids, macrophages, smooth muscle cells and collagen fibers.

Our findings suggest that females were affected to a lesser extent by arsenic compared to their male counterparts with regards to the size of atherosclerotic plaques. In females, there were no meaningful changes in the plaque size in the aortic sinus post water and diet treatment, however there were minimal changes in the plaque constituents. While macrophage, smooth muscle cell and collagen levels were unaltered, there was a significant increase in the lipid levels in the absence of high fat diet.

Together these findings reveal that the impact of arsenic on the atherosclerotic plaque is different in females than in males, supporting the hypothesis that there are sex- and diet-dependent changes in arsenic-induced atherosclerotic plaques. The work here highlights the need for targeted strategies to understand and mitigate arsenic-induced cardiovascular diseases. Further examination of plaque components through single cell multiome sequencing and Phenocycler imaging system might contribute to the understanding of immune cell diversity within atherosclerotic plaques as well as help the identification of crucial biomarkers for early detection of arsenic induced atherosclerosis in both sexes.

Résumé

L'arsenic est identifié comme l'un des contaminants chimiques les plus importants au monde par l'Organisation mondiale de la santé (OMS). Pourtant, des millions de personnes sont toujours exposées quotidiennement à l'arsenic via l'air, le sol et les eaux souterraines contaminés par des sources naturelles et anthropiques. Même si les concentrations d'arsenic sont fortement réglementées sur le lieu de travail, dans l'agriculture et dans l'eau potable municipale, l'exposition à l'arsenic reste une menace importante pour la santé humaine. L'exposition à l'arsenic provoque une myriade d'effets néfastes sur la santé, tels que des troubles neurologiques et cutanés, divers cancers et des maladies cardiovasculaires telles que l'athérosclérose. L'athérosclérose est à la fois une maladie cardiovasculaire et une maladie immunitaire principalement associée au rétrécissement et au durcissement des artères dus à l'accumulation de fibro-graisse impliquant diverses cellules immunitaires, le collagène et le cholestérol oxydé. En raison de la teneur en cholestérol, un régime riche en graisses est un contributeur connu de l'athérosclérose. Cependant, il existe des dimorphismes sexuels entre les hommes et les femmes en matière de développement de l'athérosclérose.

Précédemment, notre laboratoire a montré que l'arsenic augmente la taille de la plaque dans le sinus aortique chez les souris mâles apolipoprotein E knockout (apoE$^{-/-}$) en l'absence d'un régime riche en graisses, tout en modifiant divers constituants de la plaque indépendamment du régime alimentaire. Alors que les niveaux de macrophages restaient constants, les niveaux de lipides augmentaient tandis que les niveaux de collagène et de cellules musculaires lisses diminuaient. Cependant, les plaques d'athérosclérose induites par l'arsenic n'ont jamais été caractérisées chez les femmes.

Ainsi, dans cette étude, nous avons étudié le rôle du sexe et de l'alimentation dans l'athérosclérose induite par l'arsenic chez les souris femelles apoE$^{-/-}$ en présence et en absence d'un régime riche en graisses en:

1) Mesurant la taille des plaques athéroscléreuses induites par l'arsenic dans le sinus aortique.
2) Mesurant les niveaux de constituants clés des plaques d'athérosclérose induites par l'arsenic dans le sinus aortique, tels que les lipides, les macrophages, les cellules musculaires lisses et les fibres de collagène.

Nos résultats suggèrent que les femmes étaient moins affectées par l'arsenic que leurs homologues masculins en ce qui concerne la taille des plaques d'athérosclérose. Chez les femmes, il n'y a eu aucun changement significatif dans la taille de la plaque dans le sinus aortique après le traitement par l'eau et le régime, mais il y a eu des changements minimes dans les constituants de la plaque. Bien que les niveaux de macrophages, de cellules musculaires lisses et de collagène soient restés intacts, il y a eu une augmentation significative des niveaux de lipides en l'absence de régime riche en graisses.

Ensemble, ces résultats révèlent que l'impact de l'arsenic sur la plaque d'athérosclérose est différent chez les femmes que chez les hommes, tout en supportant l'hypothèse selon laquelle il existe des changements dépendants du sexe et du régime alimentaire dans les plaques d'athérosclérose induites par l'arsenic. Les travaux mentionnés ici mettent en évidence la nécessité de stratégies ciblées pour comprendre et atténuer les maladies cardiovasculaires induites par l'arsenic. Un examen plus approfondi des composants de la plaque grâce au séquençage multiome unicellulaire et

au système d'imagerie Phenocycler pourrait contribuer à la compréhension de la diversité des cellules immunitaires au sein des plaques athéroscléreuses ainsi qu'aider à l'identification de biomarqueurs cruciaux pour la détection précoce de l'athérosclérose induite par l'arsenic chez les deux sexes.

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Contribution of Authors

Ayse Nazli Zengin – performed all experiments, imaging, analysis, and thesis writing

Kiran Makhani – supervision on exposure and cryosection

Nivetha Subramaniam – supervision on various staining techniques (oil red O, picrosirius red, MOMA-2, a-SMA)

Natascha Gagnon – breeding and managing the apoE$^{-/-}$ colony, assistance with experiments

Koren K Mann – funding acquisition, experimental design, and project supervision

Chapter 1: Introduction

1.1 Arsenic

Arsenic is a ubiquitous metalloid initially discovered by German intellectual Albertus Magnus in 1250 (Smith, 2022). However, the first instructions to prepare pure arsenic were written by Paracelsus also known as the father of modern toxicology, only in 1500s, almost 300 years later its discovery (Smith, 2022). Due to its subtlety and potency, arsenic became very notorious as a homicidal and suicidal agent since it was discreet yet highly toxic (Bartrip, 1992). Its involvement in many high profile murders in medieval times quickly bestowed upon arsenic the title of “king of poisons” and the “poison of kings” (Vahidnia et al., 2007). In contrast, arsenic was also used as a therapeutic agent throughout history. Examples include Fowler’s solution, which was used to treat diseases such as psoriasis and syphilis, or as arsenic trioxide to treat acute myeloid leukemia (Hughes et al., 2011; Rust & Soignet, 2001; Scheindlin, 2005; Zhang et al., 2001). Nevertheless, today, it is considered as a toxic chemical threat that is internationally recognized. Arsenic is the most significant chemical contaminant according to both World Health Organization’s (WHO) top 10 chemicals of major public health concern and Agency for Toxic Substances and Disease Registry’s (ASTDR) substance priority list (Agency for Toxic Substances and Disease Registry, 2023a; World Health Organization, 2020a).

1.1.1 Distribution of arsenic across geographic space and human exposure to arsenic

Arsenic is a naturally occurring element and ubiquitously distributed to almost all corners of the globe (Fatoki & Badmus, 2022). On average, earth's outermost layer, also known as the crust, has a natural concentration of 1.5 to 2 parts per million (ppm) arsenic, making it the 20th most abundant element in the crust (National Research Council (US) Committee on Medical and Biological Effects of Environmental Pollutants, 1977b). Due its physical and chemical properties, arsenic can be found in many forms, such as alloys (mixtures of metals), sulfosalts, and minerals, making it more readily available in many different environments, such as water systems and rock sediments (National Research Council (US) Committee on Medical and Biological Effects of Environmental Pollutants, 1977c). Through geological processes such as volcanic eruptions, forest fires and weathering of arsenic rich rocks, arsenic can be released into air, soil, and water leading to human exposure through inhalation, ingestion and dermal exposure (Nriagu et al., 2007). During a volcanic eruption, with carbon dioxide and sulfur dioxide, large amounts of heavy metals get released into the air propelled with volcanic ash (Žuškin et al., 2007). Arsenic typically concentrates on the surface of volcanic ash and gets released into aquatic systems when the volcanic ash comes into contact with water, contaminating it (Bia et al., 2015). Contaminated water creates a potential for human exposure if used as irrigation and or as drinking water.

Another natural source that is important for arsenic contamination is weathering of arsenic rich rocks and soils that leads to contamination of groundwater, often at high concentrations (Agency for Toxic Substances and Disease Registry, 2007). According to

the WHO, the top 10 countries with contaminated groundwater with naturally high arsenic concentrations are Cambodia, Chile, China, India, Mexico, Pakistan, Vietnam, Bangladesh, and the United States of America (World Health Organization, 2022). It is estimated that 140 million people in 70 different countries have been exposed to groundwater contaminated with arsenic above the maximum allowable limit set by WHO (World Health Organization, 2022).

Of course, this number is not only due to arsenic contamination from natural sources but also from human activity. In fact, arsenic contamination is primarily due to anthropogenic sources such as metal mining and improper disposal of associated waste, coal combustion, wood preservatives and use of arsenic based agricultural pesticides (Agency for Toxic Substances and Disease Registry, 2007). Arsenic containing pesticides often contaminate the soil and result in arsenic accumulation in plants (Agency for Toxic Substances and Disease Registry, 2007). In addition to arsenic based pesticides, the use of arsenic contaminated water for irrigation results in arsenic accumulation in grains, such as rice, and vegetables, such as tomatoes and cabbage (Sandil et al., 2021; Upadhyay & et al., 2019). Moreover, wildlife exposure to arsenic is a public health concern (Shaw & et al., 2007). Aquatic organisms can bioaccumulate arsenic and the arsenic concentrations increase moving from bottom feeders to predatory fish (Agency for Toxic Substances and Disease Registry, 2007). Consumption of contaminated sea food, produce and drinking water due to both natural and anthropogenic sources are the key elements of human exposure (Goyanna et al., 2023; World Health Organization, 2022).

1.1.2 Physical and chemical properties of arsenic

Physical properties: In the periodic table, arsenic has the symbol As and the atomic number of 33. It is a member of the group pnictogens, formerly known as group V or Va, alongside nitrogen, phosphorus, antimony, bismuth and moscovium. In nature, it can be found as yellow, black or grey arsenic (International Labour Office, 1971; Pichon, 2013). Grey arsenic is the most common among the three allotropes and is also the most stable in room temperature. In contrast, yellow arsenic is made out of As_4 molecules (Yoshiasa et al., 2019). Black arsenic is amorphous and the rarest allotrope (Antonatos et al., 2020; Yoshiasa et al., 2019).

Chemical properties: Arsenic has many oxidation states such as -3, 0, +3, and +5 known as arsenides, elemental arsenic, arsenites, and arsenates, respectively (World Health Organization, 2020b). Although arsenic can be mostly found bound to sulfur and oxygen in the nature, due to its high reactivity and wide range of oxidative states, arsenic can also be found bound to many metals such as copper and lead, forming alloys (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006; O'Day, 2006).

Moreover, arsenicals have different solubilities depending on their ionic surroundings, binding partners, pH, and temperature (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004) . While some forms of arsenicals such as arsenic sulfides are insoluble, arsenites are soluble in water (Habashi, 2001; National Research Council (US) Committee on Medical and Biological Effects of Environmental Pollutants, 1977a). Due to different solubility profiles, the most common oxidation states in nature are trivalent and pentavalent forms, which are also considered highly toxic (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004). Arsenic can inhibit

essential cellular mechanisms affecting cell survival (Tam et al., 2020), but has many additional mechanisms of toxicity, some of which remain questions in the field.

1.1.3 Pharmacokinetics of arsenic in mammals

Arsenic is a toxic element and causes health problems upon exposure (World Health Organization, 2022). Like many toxicants, arsenic goes through several pharmacokinetic steps to be eliminated (Agency for Toxic Substances and Disease Registry, 2023b). Many mammals, including humans, biomethylate arsenic to eliminate it from their bodies as a mechanism of detoxification, which may take place primarily in the liver and excreted through the kidneys. Bioavailability of arsenic depends on the type of arsenic and exposure (acute or chronic) however literature shows that 45-85% of orally exposed inorganic arsenic is excreted within three days in urine in humans (Apostoli et al., 1999; Buchet et al., 1981; Crecelius, 1977; Mappes, 1977; Tam et al., 1979). This process is even faster in mice with 90% clearance within two days (Hughes et al., 2003; Vahter, 1981). Arsenic does not accumulate in any vital organs, yet trace arsenic can be found in hair, skin, and nails up to two to four weeks after the exposure has come to an end (Lansdown, 1995).

1.1.3.1 Absorption

Humans are exposed to arsenic through inhalation, dermal contact, and ingestion (Agency for Toxic Substances and Disease Registry, 2023d). The small intestine is the major site of absorption (Kellett et al., 2022). However, there are no arsenic specific cellular uptake mechanisms as the body has for essential nutrients, further supporting the idea that arsenic does not provide an essential function for living organisms (El-Ghiaty &

El-Kadi, 2023). Therefore, arsenic uses already existing channels to enter and exit cells. Glycerol channels, such as aquaporins, are membrane bound proteins that are responsible for transporting water and small neutral solutes, across plasma membranes (Bhattacharjee et al., 2009). Isoforms AQP3, 7 and 9 have been reported to bidirectionally carry arsenite in mammals including humans (Liu et al., 2002). Furthermore, when AQP3 and 9 were overexpressed in human leukemia cells, arsenic influx was increased, suggesting the involvement of aforementioned transporters in arsenic transportation (Bhattacharjee et al., 2004). In addition to these transporters, arsenic can also use GLUT1, a glucose permease, to be imported or exported (Liu et al., 2006). Monomethylarsonous acid (MMA^{III}) and arsenic trioxide (As_2O_3) use glucose/fructose transporters to enter heart and brain cells, where GLUT1 expression is high (Jiang et al., 2010; Liu et al., 2006). In addition, inhibition studies done using phloretin and copper sulfate to block GLUT transporters and aquaporins resulted in a reduced permeability of arsenite *in vitro* (Calatayud et al., 2012). Moreover, arsenate ions have a striking similarity to that of phosphates meaning that they can enter a cell using phosphate transporters such as PIT1 and PIT2 due to structural resemblance. (Calatayud et al., 2012; Delides & Iatropoulos, 1979).

1.1.3.2 Distribution

The distribution of arsenic is mostly done by the circulatory system (Agency for Toxic Substances and Disease Registry, 2007). Arsenic can either travel in an unbound state in the plasma or hitchhike by binding to blood cells (Kumana et al., 2002; Shi et al., 2019). White and red blood cells, among other cells with arsenic reducing abilities can take up

arsenic and transport it throughout the blood stream to be metabolized (Winski & Carter, 1995).

1.1.3.3 Metabolism

Different phylogenetic groups have different detoxification strategies to eliminate arsenic such as efflux transporters, sequestration, metal binding proteins and biotransformation (Garbinski et al., 2019; Guo et al., 2012; Rahman & De Ley, 2017). Arsenic methylation is traditionally considered as the primary way to detoxify arsenic in mammals, including humans (El-Ghiaty & El-Kadi, 2023). Methylated arsenic metabolites get eliminated much faster than inorganic arsenic (Buchet et al., 1981). The methylation reaction is catalyzed by an enzyme called arsenic 3 methyltransferase (AS3MT) and the deletion of AS3MT results in the subsequent accumulation of inorganic arsenic (Chen et al., 2011; Douillet et al., 2017; Drobna et al., 2009; K. Huang et al., 2018; M. C. Huang et al., 2018; Thomas et al., 2001; Yokohira et al., 2010). Even though methylated inorganic arsenic metabolites are well established, there is still a debate in the scientific community on the precise sequence of reactions and enzymes involved in the process (El-Ghiaty & El-Kadi, 2023). There are four main proposed pathways (figure 1):

1) **Challenger's pathway** (Challenger, 1945): In 1945, Frederick Challenger proposed that inorganic arsenic metabolism was primarily alternating redox reactions until a less toxic metabolite that can be easily eliminated is formed. In the Challenger pathway, glutathione S-transferase omega-1 (GSTO1) uses glutathione (GSH) as a reducing agent and AS3MT also known as Cyt19, uses S-adenosylmethionine (SAM) as a methyl donor to form mono-, di-, and tri-methylated metabolites. Arsenate (V) is reduced to arsenite (III) and

subsequently, methylated to monomethylarsonic acid (MMA^{V}), which also is reduced to monomethylarsonous acid (MMA^{III}). Followed by a second methylation step, MMA^{III} turns into dimethylarsinic acid (DMA^{V}), which is reduced into dimethylarsenous acid (DMA^{III}). With a third methylation step, DMA^{III} is methylated into trimethyl arsine oxide ($TMAO^{V}$), which eventually is reduced into trimethyl arsine (TMA^{III})

2) **Hayakawa's pathway** (Hayakawa et al., 2005): In 2005, Toru Hayakawa suggested that inorganic arsenic metabolism involves arsenic-glutathione (As-GSH) complexes. His studies showed that when both arsenite (III) and GSH are present, an As-GSH complex known as arsenic triglutathione (ATG) forms without the help of an enzyme. Moreover, he hypothesized that monomethylarsonic diglutathione (MADG) was methylated to dimethylasrinic glutathione (DMAG) by AS3MT, but MMA^{III} was not metabolized into DMA^{V} . However, MADG and DMAG were hydrolyzed and oxidized into MMA^{V} and DMA^{V} only when GSH levels were low. His work suggests that inorganic arsenic metabolism is through ATG and MADG instead of arsenite (III) and MMA^{III}.
3) **Naranmandura's pathway** (Naranmandura et al., 2006): In 2006, Hua Naranmandura suggested that proteins bind to arsenites during reductive methylation reactions in order to be eliminated. His studies done on rats revealed that 80% of arsenic in soluble sediments of organs were associated with high molecular weight (HMW) proteins. When these arsenic – HMW complexes were oxidized with hydrogen peroxide, the arsenic that was dissociated from HMWs were in arsenate (V) form such as MMA^{V} and DMA^{V}

meaning that only arsenites (III) bind to HMWs and the methylation process occurs through simultaneous reduction in the presence of GSH rather than sequential oxidative methylation.

4) **Stýblo's pathway** (Stýblo et al., 2021): In 2021, Miroslav Stýblo suggested a model that includes both a pathway for arsenic entry/exit from the cell and a pathway for arsenic methylation within the cell. The main difference of this pathway lies in the fact that arsenic is methylated without changing the oxidation state. The oxidation state can change intracellularly after methylation step. At any step of the pathway, different arsenic species can enter or exit the cell.

Until now, arsenic metabolism was discussed as a strategy to convert toxic arsenicals into lesser toxic forms to facilitate its clearance, however, it is wise to consider that xenobiotic transformation may result in generation of intermediates with higher toxicities than the parent compound (El-Ghiaty & El-Kadi, 2023; Stýblo et al., 2021). Literature shows that MAs^{III} and $DMAs^{III}$, the trivalent methylated forms of inorganic arsenic, are more toxic than inorganic trivalent arsenic suggesting that arsenic methylation may not only be a way of arsenic detoxification but also a way of arsenic bioactivation (Stýblo et al., 2021). More research should be done on arsenic metabolism to explore this dual purpose of arsenic methylation both as a catalyst to arsenic elimination but also a generator of more toxic arsenic metabolites.

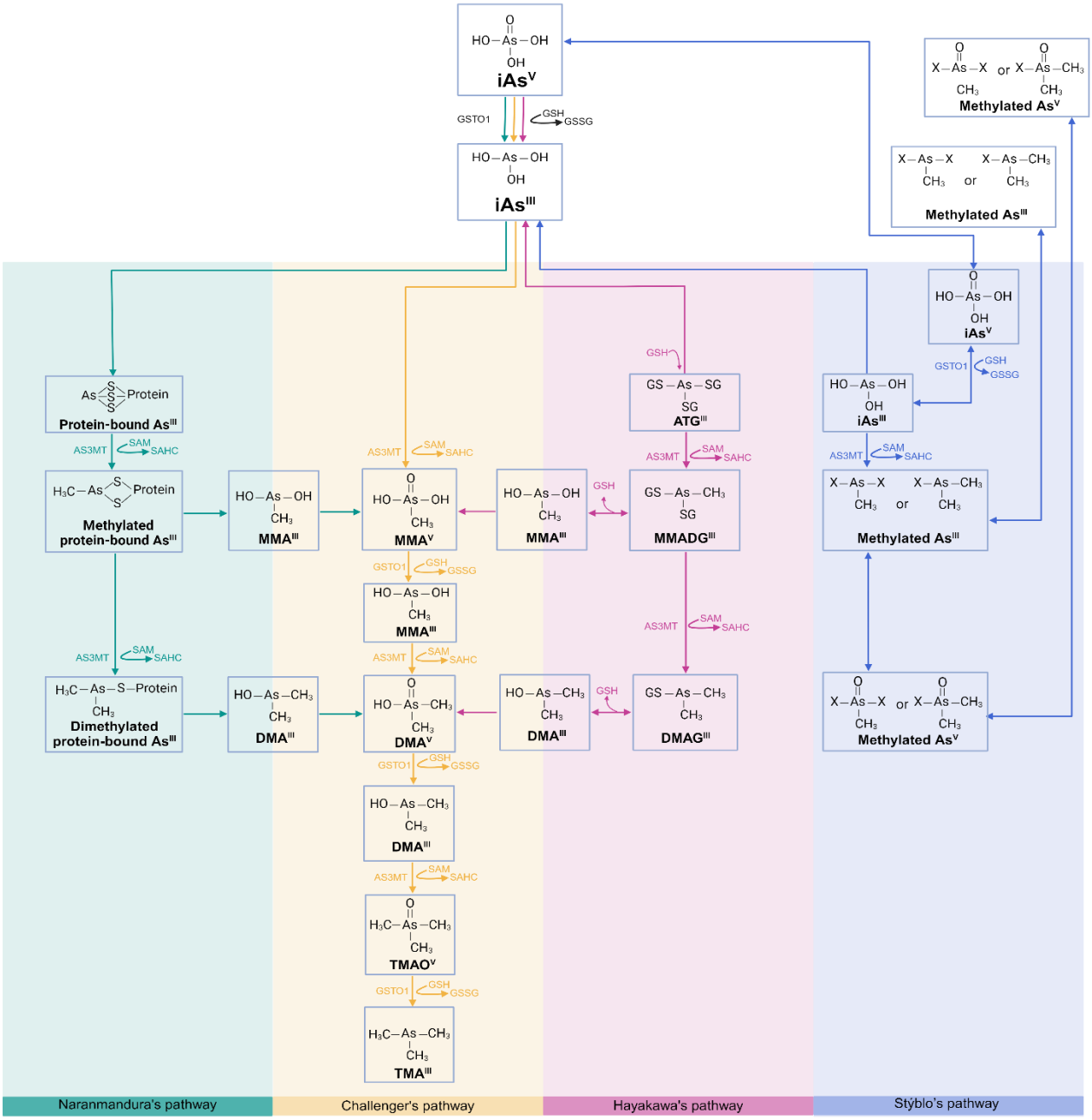

Figure 1: Schematic of the 4 main pathways of arsenic biotransformation in mammals. Figure generated using Biorender and adapted from El-Ghiaty et al., 2023 and Styblo et al., 2021. The abbreviations as follows: **iAs^{V}** (inorganic pentavalent arsenic), **GSTO1** (glutathione S-transferase omega-1), **GSH** (reduced glutathione), **GSSG** (oxidized glutathione), iAs^{III} (inorganic trivalent arsenic), **AS3MT** (arsenic-3-methyltransferase), **SAM** (S-adenosylmethionine), **SAHC** (S-adenosylhomocysteine), **ATG^{III}** (arsenic triglutathione), **MMA^{III}** (monomethylarsenous acid), **MMA^{V}** (monomethylarsonic acid), **MMADG^{III}** (monomethylarsonous acid diglutathione), **DMA^{III}** (dimethylarsinous acid), **DMA^{V}** (dimethylarsinic acid), **DMAG^{III}** (dimethylarsinous acid glutathione), **TMAO^{V}** (trimethylasine oxide), **TMA^{III}** (trimethylarsine). X in Stýblo's pathway stands for any chemical group.

1.1.3.4 Elimination

Arsenic is eliminated from the body through the kidneys (Agency for Toxic Substances and Disease Registry, 2023b). Mostly pentavalent arsenic and its methylated forms are excreted in urine (Agency for Toxic Substances and Disease Registry, 2007). DMA^{V} was thought to be the final product of arsenic methylation due to its lower toxicity and quick elimination (Aposhian & Aposhian, 2006; Cullen, 2014; El-Ghiaty & El-Kadi, 2023). However, recent studies shed light on urinary arsenic metabolites demonstrating that there are also trivalent methylated arsenic intermediates present in urine, which are less stable and more toxic than their pentavalent counterparts (Borak & Hosgood, 2007; Cohen et al., 2006; Moe et al., 2016; Naranmandura et al., 2011). The distribution of arsenic compounds in human urine are as follows though these ratios heavily dependent on the dose, duration, and the exposure type: 10-30% inorganic arsenicals such as arsenite and arsenate, 10-20% monomethylated arsenicals such as MMA^{III} and MMA^{V} and 60-80% demethylated arsenicals MMA^{V} and DMA^{V} (Hernandez & Marcos, 2008; Huang et al., 2009; Steinmaus et al., 2005; Vahter, 2000).

On a cellular level, arsenic is effluxed by the adenosine triphosphate (ATP) binding cassette (ABCC) transporters such as ABCC1, 2, and 4 (Banerjee et al., 2014; Carew et al., 2011; Kala et al., 2000). In vivo studies done on plants showed increased toxicity due to intracellular arsenic accumulation in plants lacking ABCC1 and ABCC2 transporters, demonstrating ABCC1 and ABCC2 is essential for arsenic export (Maciaszczyk-Dziubinska et al., 2012). However, the transportation of arsenic metabolites for elimination from the liver to the blood does not involve ABCC1 or ABCC2 but ABCC4 (Banerjee et al., 2014). ABCC4 is expressed both in the hepatocytes and renal tubule cells, facilitating

the elimination of arsenic metabolites such as DMA^V, which is the most prevalent arsenic metabolite in human urine (Banerjee et al., 2014).

Elimination of arsenic happens fairly rapidly. Arsenic is excreted within a few days post exposure in humans and this elimination process is even faster in mice (Apostoli et al., 1999; Buchet et al., 1981; Crecelius, 1977; Hughes et al., 2003; Mappes, 1977; Tam et al., 1979; Vahter, 1981). However, a study involving human volunteers and a one-time intravenous injection of radiolabeled trivalent inorganic arsenic showed that lesser amounts of arsenic can be detected in the urine up to 2 weeks after the injection (Agency for Toxic Substances and Disease Registry, 2007). Other routes of arsenic elimination include sweating, peeling skin, defecation, sheading hair and clipping nails (Agency for Toxic Substances and Disease Registry, 2023b).

1.1.4 Arsenic Toxicity

Arsenic toxicity can be acute (short term) or chronic (long term) depending on the duration of the exposure (Ratnaike, 2003). The severity and the symptoms of the toxicity are closely related to the species of arsenic to which one is exposed, as well as the dose, duration, and route of exposure (Hong et al., 2014; Sharma & Sohn, 2009). Chronic arsenic exposure is more common than acute arsenic exposure globally (Cleveland Clinic, 2023).

1.1.4.1 Acute toxicity

Acute arsenic toxicity is the combination of adverse health effects induced by a short term arsenic exposure such as hours to days (Sobanska et al., 2014). Though the symptoms change depending on the dose, acute arsenic toxicity can present itself in many ways. An

extensive list of symptoms of acute arsenic toxicity can be found in Table 1 (Agency for Toxic Substances and Disease Registry, 2023c). Most of these symptoms present themselves relatively soon after exposure (Kuivenhoven & Mason, 2023). However, in some cases, symptoms such as neuropathy and Mee's lines, horizontal white lines on nails, could be delayed by several weeks to months (Agency for Toxic Substances and Disease Registry, 2023c).

A low dose, acute arsenic exposure can result in mostly gastrointestinal symptoms such as diarrhea, nausea and vomiting and can resolve itself without treatment within 12 hours however, more severe cases due to higher doses may end in death due to cardiovascular failure and hypovolemic shock (Kingston et al., 1993) (Agency for Toxic Substances and Disease Registry, 2023c). 0.6mg/kg/day of arsenic trioxide, which is one of the components of the combination therapy for acute myeloid leukemia, is considered a fatal dose for humans upon ingestion over a short period of time such as 14 days (Agency for Toxic Substances and Disease Registry, 2007; Helso et al., 2020).

Table 1: Symptoms of Acute Arsenic Toxicity	
Gastrointestinal symptoms	Breath smelling like garlic, abdominal pain, nausea, vomiting, dehydration, thirst, anorexia, blood or rice water diarrhea, dysphagia, and heartburn.
Dermal symptoms	Dermatitis, vesiculation, melanosis and Mee's lines
Respiratory symptoms	Pulmonary edema, tracheobronchitis, bronchial pneumonia, perforation in the septum, irritation in the nasal mucosa as well as the pharynx, larynx, and the bronchi.
Neurologic symptoms	Tremor, disorientation, encephalopathy, delirium, lethargy, weakness, headache, autonomic neuropathy with sweating and flushing, neuritis, hyperpyrexia, stupor, paralysis, neuropathy, and coma.
Cardiovascular symptoms	T wave inversion, arrhythmia, congestive heart failure, hypovolemic shock, and hypotension

Renal symptoms	Uremia, renal cortical and acute tubular necrosis, glycosuria, leukocyturia, oliguria, proteinuria, and hematuria
Hematologic symptoms	Leukopenia, anemia, thrombocytopenia, intravascular coagulation, and suppression of the bone marrow.
Hepatic symptoms	Necrosis, cholangitis and cholecystitis, fat infiltration, congestion, and increased liver enzymes

1.1.4.2 Chronic toxicity

Chronic arsenic toxicity is the combination of adverse health effects induced by arsenic exposure following multiple exposures over a prolonged period of time such as lifelong exposures (Hurst, 2014). Chronic arsenic exposure is currently affecting 100 million people globally, mostly through contaminated drinking water (Ng et al., 2003). Sustained arsenic toxicity causes diverse symptoms affecting multiple systems and an extensive list of symptoms of chronic arsenic toxicity can be found in Table 2 (Agency for Toxic Substances and Disease Registry, 2023c). Symptoms such as hyperkeratosis and skin hyperpigmentation could present themselves delayed after the arsenic exposure (Agency for Toxic Substances and Disease Registry, 2023c).

Table 2: Symptoms of Chronic Arsenic Toxicity	
Gastrointestinal symptoms	Esophagitis, gastritis, colitis, abdominal discomfort, anorexia, malabsorption, and weight loss.
Dermal symptoms	Hyperpigmentation, skin lesions, hyperkeratosis, desquamation, and skin cancer.
Respiratory symptoms	Tracheobronchitis, pulmonary insufficiency, rhino-pharyngo-laryngitis, chronic restrictive and obstructive disease, and lung cancer
Neurologic symptoms	Neuropathy, polyneuritis, motor paralysis, hearing loss, intellectual disabilities, encephalopathy, symmetrical peripheral polyneuropathy, electromyographic abnormalities.
Cardiovascular symptoms	Arrythmias, Blackfoot disease, ischemic heart disease, carotid atherosclerosis, microcirculation

	abnormalities, pericarditis, Raynaud's syndrome, acrocyanosis, cerebral infarction, and hypertension.
Renal symptoms	Proteinuria, hematuria, acute renal failure, and bladder cancer.
Hematologic symptoms	Aplastic anemia, leukopenia, impaired folate metabolism, karyorrhexis, bone marrow hypoplasia, and thrombocytopenia.
Hepatic symptoms	Enlarged and tender liver increased hepatic enzymes, cirrhosis, portal hypertension without cirrhosis, and fatty degeneration.
Endocrine symptoms	Diabetes mellitus.

1.1.5 Arsenic-induced cardiovascular outcomes

Arsenic exposure causes many adverse health problems including cardiovascular diseases which are the diseases of the heart and vascular system (World Health Organization, 2020c). Cardiovascular diseases claim almost 18 million lives each year (World Health Organization, 2024). Ischemic heart disease-associated deaths increased from 2 million deaths/year to 8.9 million deaths/year in 19 years from 2000 to 2019, becoming the leading cause of death worldwide (World Health Organization, 2020c). Iin the U.S., which is one of the top 10 countries with naturally high arsenic levels in groundwater, this trend has likely been occurring for the last century (American Heart Association, 2024; World Health Organization, 2022). Moreover, epidemiological studies demonstrate that increased morbidity and mortality due to cardiovascular diseases have been linked to chronic arsenic exposure. In fact, a statement issued by the American Heart Association (AHA) in 2023, revealed that low to moderate levels of chronic exposure of heavy metals, including arsenic, increases the risk of cardiovascular diseases (Lamas et al., 2023). Human data from Taiwan shows that exposure to groundwater with elevated levels of arsenic increases the risks of hypertension, ischemic

heart disease, and atherosclerosis (Chen et al., 1995; Hsueh et al., 1998; Wang et al., 2002).

1.2 Atherosclerosis

Atherosclerosis is a systemic disease that involves both the cardiovascular and the immune system (Lusis, 2000; Yuan et al., 2019). It is often marked by plaque buildup in the large arteries, resulting in the stiffening and narrowing (Shahjehan & Bhutta, 2023). The word atherosclerosis comes from the Greek words “athere” meaning “gruel” and “skleros” meaning “hard”, indicating the accumulation of fatty substances hardening and thickening the arteries (Marques et al., 2021). Even though atherosclerosis alone is benign, it is the root cause of many life-threatening cardiovascular events, such as ischemic heart disease and stroke, the top two leading causes of death worldwide for the past two decades (Badimon et al., 2012; Lusis, 2000; Sodhi & Brown, 2019; World Health Organization, 2020c) .

1.2.1 Etiology

Like most cardiovascular diseases, atherosclerosis is a multifactorial disease with many modifiable (lifestyle, diet, smoking, and environmental exposures) and nonmodifiable risk factors (age, sex, and genetics) (Pan et al., 2024).

<u>Modifiable risk factors:</u>

1) Lifestyle: Regular exercise increases blood flow and sheer stress which regulates vascular homeostasis by decreasing the reactive oxygen species (ROS) production (Wang et al., 1993). This leads to an improvement of endothelial function by increasing the bioavailability of nitric oxide in endothelial

cells, counteracting atherosclerosis development both by increasing vasodilation and reducing oxidative stress (Niebauer & Cooke, 1996; Wang et al., 1993).

2) Diet: High fat diets are risk factors for atherosclerosis (Nishina et al., 1993; Zhang et al., 1994). Excessive amount of saturated fats results in the accumulation of low-density lipoproteins (LDLs) in the artery walls leading to atherosclerosis (Ruuth et al., 2021). Moreover, regular alcohol consumption is also a risk factor (Kiechl et al., 1998). Epidemiological data shows that heavy alcohol consumers have a higher risk of developing atherosclerosis compared to those who do not drink, even surpassing the risk caused by smoking (Kiechl et al., 1998).
3) Smoking: Cigarette smoking causes morphological and biochemical dysfunction in endothelial cells, which is a key initiator of atherosclerosis development (Michael Pittilo, 2000; Ross, 1993).
4) Environmental exposures: Exposures to metals, such as lead, mercury, and arsenic, leads to atherosclerosis by causing a state of chronic inflammation (Lamas et al., 2023).

<u>Non-modifiable risk factors:</u>

1) Age and sex: With age, serum cholesterol levels increase, contributing to the development of atherosclerosis (Beckett et al., 2000). However, this increase happens earlier in males and then decreases, while female serum cholesterols do not increase until later in life then rapidly increase after menopause (Felix-Redondo et al., 2013). Moreover, the female sex hormone estrogen has

cardioprotective effects (Moolman, 2006). Literature showed that ovariectomy resulted in a loss of cardioprotection while chronic estrogen therapy rescued the phenotype in rats, showing that estrogen plays a role in protecting the cardiovascular system (Zhai et al., 2000).

2) Genetics: Genetic mutations causing dyslipidemia and hyperglycemia are initiators of inflammation thus they are also risk factors for atherosclerosis (Jackson et al., 2018). Moreover, individuals predisposed to diabetes and hypertension are also at risk (Petrie et al., 2018). These conditions are also a risk factor for the development of atherosclerosis (Poznyak et al., 2022; Ye et al., 2022) .

Although non-modifiable risk factors will remain a concern, controlling the modifiable risk factors could prevent the development of atherosclerosis by not building on the existing risk.

1.2.2 Pathophysiology

The switch from healthy to diseased state does not happen overnight. Accumulation of pathogenic stimuli shifts the balance from healthy to sick gradually. In the case of atherosclerosis, excess plasma cholesterol triggers lipid retention in the intima (Rafieian-Kopaei et al., 2014). Subsequent lesion formation coupled with a chronic inflammatory environment result in the development and progression of atherosclerosis (Libby, 2021). This process takes place over three main stages known as lesion initiation, fatty streak formation, formation of early atheroma and atherosclerotic plaques with fibrous cap (figure 2) (Steinl & Kaufmann, 2015). It can start as early as childhood and become

symptomatic later in life depending on genetic and environmental factors (Luca et al., 2023). This process could also lead to plaque rupture and a subsequent thrombosis however it is not seen in all atherosclerosis cases (Asada et al., 2020).

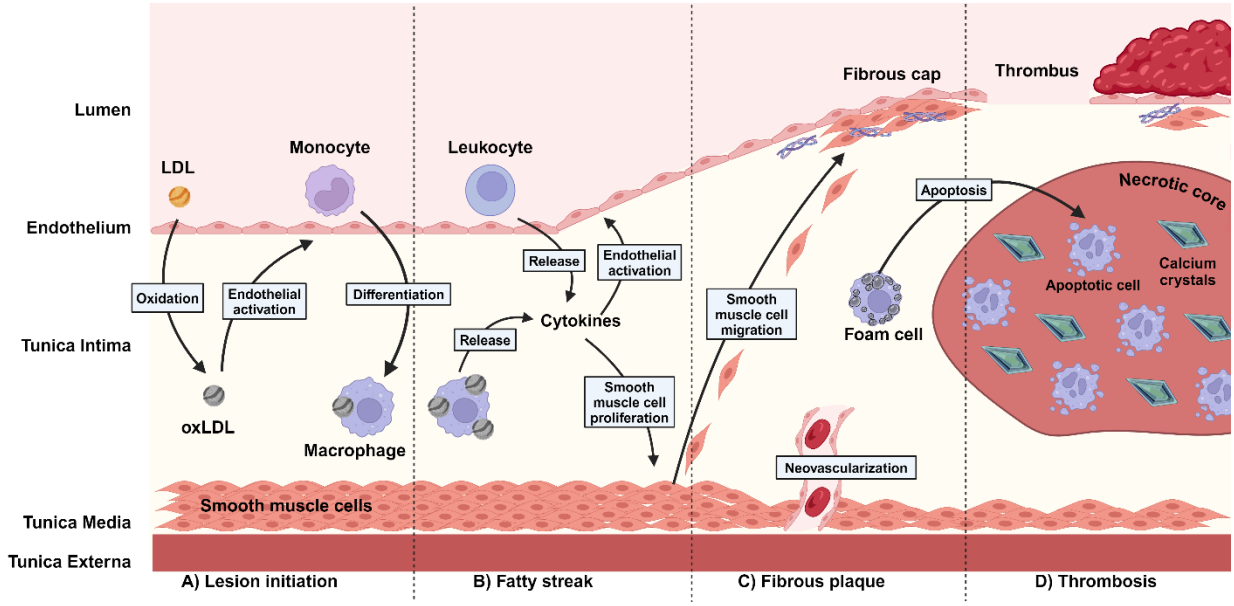

Figure 2: Stages of atherosclerotic plaque development. Figure generated using Biorender and adapted from Steinl & Kaufmann, 2015.

1.2.2.1 Lesion initiation

The first step of atherosclerosis is the lesion initiation, and it can be grouped in two stages: 1) endothelial dysfunction and LDL trapping; and 2) oxidation of LDLs and activation of leukocytes.

Endothelial dysfunction and LDL trapping: The artery wall has 3 main layers known as the tunica externa, tunica media, and tunica intima (Saxton et al., 2023). The outermost layer of the artery (furthest from the lumen) is called tunica externa or also known as tunica adventitia and is made of connective tissue and nerves (Saxton et al., 2023). The middle layer is called tunica media, and it is primarily consistent of smooth muscle cells (Saxton et al., 2023). The innermost layer is called the tunica intima, and it is formed by a

monolayer of endothelial cells surrounded by connective tissue known as the basement layer (Brown et al., 2017). Endothelial cells in the tunica interna are the first barrier between the circulating blood and the tissues, acting as a sensor to perceive any chemical and mechanical changes such as changes in coagulation, vessel tone and inflammation (Feletou et al., 2008; van Hinsbergh, 2012).

Mechanical changes such as the changes in blood flow are linked to atherosclerotic lesion formation (Back et al., 2013). Endothelial cell alignment changes depending on the blood flow, which depends on the shape of the vessel (Zhou et al., 2023). Straight parts of the arteries experience a laminar flow with a moderate sheer stress, whereas the blood flow at bifurcation sites, branches or bends, like the aortic arch, is disrupted resulting in turbulent flow with a low sheer stress (figure 3) (Chiu & Chien, 2011). The changes in blood flow result in morphological changes in endothelial cells and promote fibro fatty build up in the branching points (Jebari-Benslaiman et al., 2022; Morley et al., 2021).

Increase in the plasma LDL levels and the subsequent inflammation are both chemical stimuli that the vessels respond to (Catapano et al., 2017; Gimbrone & Garcia-Cardena, 2016). LDL molecules cannot pass through endothelial junctions, but they can be endocytosed to balance the plasma and intracellular levels of LDLs, which happens in a healthy state (Goldstein & Brown, 2009; Mundi et al., 2018). When there are unhealthy amounts of plasma LDLs, many internalized LDL molecules get trapped into the intima, forming lesions and oxidative stress (Batty et al., 2022).

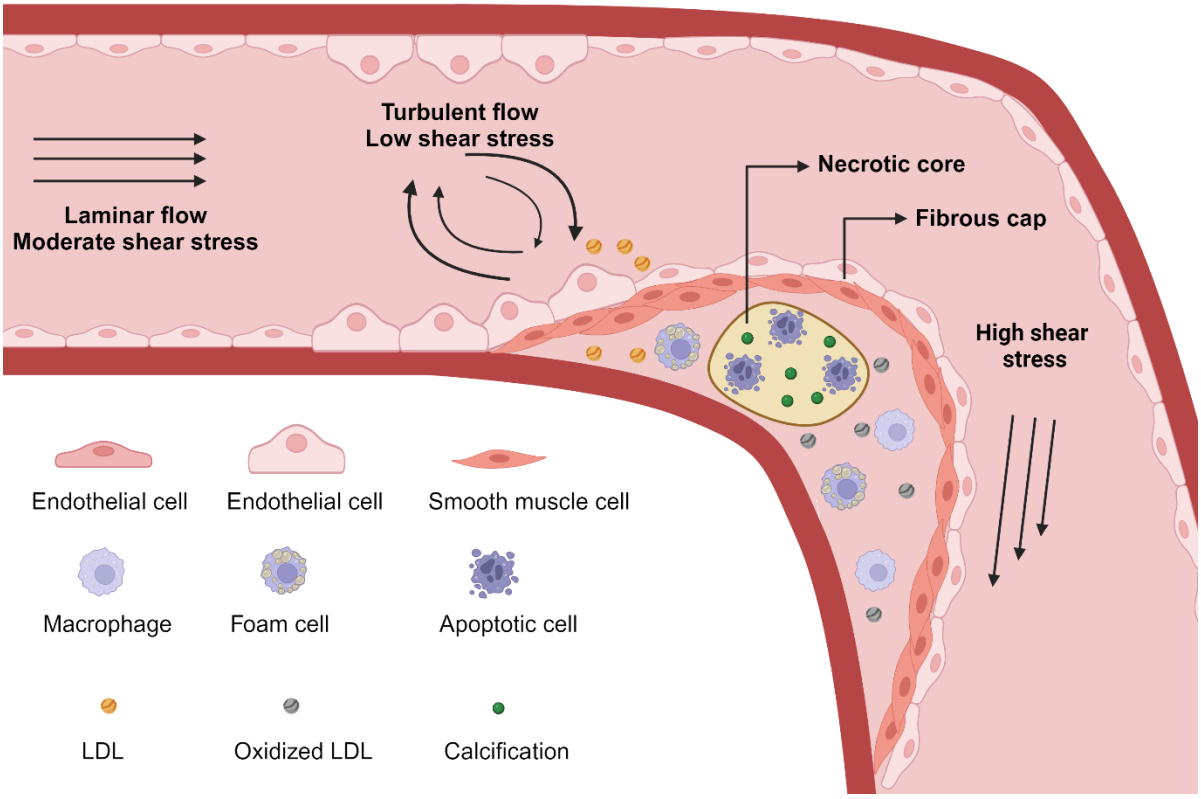

Figure 3: Schematic of how blood flow and shear stress affect atherosclerosis development. Figure generated using Biorender and adapted from Jebari-Benslaiman and colleagues.

Endothelial cells are responsible for modulating muscle tone, coagulation, and inflammation (Feletou et al., 2008; van Hinsbergh, 2012). They maintain the homeostasis by releasing both vasodilators and vasoconstrictors, coagulants, anticoagulants as well as immunomodulatory chemicals (Kang & Kishimoto, 2021; Pober & Sessa, 2007; Sandoo et al., 2010; Yau et al., 2015). Once there are chemical and mechanical stressors, the homeostasis is disrupted resulting in endothelial dysfunction (Katoh, 2023; Montiel et al., 2022).

Oxidation of LDLs and activation of leukocytes: The trapped LDLs get oxidized in the subendothelial space by free radicals (Batty et al., 2022). Chemokines and adhesion molecules expressed by the dysfunctional endothelial cells mediate the recruitment of plasma leukocytes, mostly monocytes, to the site of inflammation (Batty et al., 2022). Monocytes start rolling on the surface of the endothelial cells and with the help of vascular cell adhesion molecule 1 (VACM-1) and intracellular adhesion molecule 1 (ICAM-1), adhere to the surface of the endothelial cells (Alon & Feigelson, 2002). Then monocytes transmigrate into the intima where they differentiate into macrophages stimulated by the cytokine macrophage colony-stimulating factor (M-CSF) and polarize into different subtypes, such as foam-like, resident-like and Thrombospondin-1 (Thbs1) positive macrophages (Chi & Melendez, 2007; Kiran Makhani et al., 2023; Rosenfeld et al., 1992). In addition, macrophages are responsible for the uptake of oxidized LDLs using their scavenger receptors (Jovinge et al., 1996). Internalized oxidized LDLs get broken down by the help of lysosomes into cholesterols (Thelen & Zoncu, 2017). Then, the endoplasmic reticulum esterifies the cholesterols to be stored as lipid droplets within the cell (Jebari-Benslaiman et al., 2022; Vance, 2022).

1.2.2.2 Formation of fatty streak

As macrophages phagocytose more oxidized LDLs, they might maximize their capacity to hold lipids and turn into foam cells (Remmerie & Scott, 2018). Moreover, macrophages also release inflammatory mediators, such as cytokines including tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) (Arango Duque & Descoteaux, 2014). Thus, recruited monocytes and leukocytes and their subsequent release of cytokines create a pro-inflammatory microenvironment which triggers the smooth muscle cells residing in

the tunica media to proliferate and migrate into the intima (Louis & Zahradka, 2010; Steinl & Kaufmann, 2015). Migrated smooth muscle cells can also phagocytose oxidized LDLs through scavenger receptors, such as cluster of differentiation 36 (CD36) and lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) (Li et al., 2023). In addition, migrated vascular smooth muscle cells have less ATP-binding cassette transporter member 1 (ABCA1) transporter than the smooth muscle cells residing in the tunica media. ABCA1 is a transporter responsible for cholesterol efflux, thus smooth muscle cells with increased lipid uptake and decreased efflux can also turn into foam cells, contributing to the formation of fatty streaks alongside proinflammatory macrophages (Dubland & Francis, 2016).

1.2.2.3 Formation of early atheroma and atherosclerotic plaque with a fibrous cap

As more lipids accumulate, the nascent fatty streak grows into an early atheroma that is an intermediary stage between fatty streaks and atherosclerotic plaques. In addition to their scavenging role, vascular smooth muscle cells also produce extracellular matrix elements, such as collagen, forming a fibrous cap (Harman & Jørgensen, 2019). The fibrous cap is predominantly made from collagen rich fibers and smooth muscle cells (Alonso-Herranz et al., 2023). The formation of the fibrous cap helps the growth of atheroma to atherosclerotic plaque by stabilizing it structurally (Alonso-Herranz et al., 2023).

However, sometimes the fibrous cap is not enough to stabilize the plaque. The formation of the necrotic core counteracts the stabilization maintained by the fibrous cap (Grootaert et al., 2016). Foam cells full of oxidized LDL undergo apoptosis and the dead cells get cleared by efferocytosis (Y. Gui et al., 2022). The size of the necrotic core increases with

increased apoptosis and impaired efferocytosis (Y. Gui et al., 2022). In addition, apoptosis results in the secretion of metalloproteinases that inhibit the fibrous cap growth further impairing the structural stability of the plaque (Levkau et al., 2002).

In later stages, atherosclerotic plaques get bone-like structures due to calcification (Akers et al., 2019). Plaque calcification starts as microcalcifications due to inflammation induced by apoptotic macrophage and smooth muscle cells when calcium mineral is deposited in atherosclerotic plaques (Neels et al., 2023; Shioi & Ikari, 2018). Depending on the location, size and shape of the calcification plaque stability can be affected (Neels et al., 2023).

1.2.2.4 Plaque rupture and thrombosis

Plaque rupture and thrombosis can be considered the last stage of atherosclerosis, however not all plaques rupture or dislodge and cause subsequent blockage. Plaques that have a thin fibrous cap and increased lipid content are known as vulnerable plaques that are prone to rupture (Hafiane, 2019). Once the fibrous cap is lost, the plaque may rupture, and the necrotic core made of dead cells can be released into the circulation causing a thrombosis, resulting in myocardial infarction and stroke (Bentzon et al., 2014a; Dutta et al., 2012; Falk et al., 2013).

1.2.3 Mouse models used in atherosclerosis research

Due to their high high-density lipoprotein (HDL) and low LDL plasma profile, wild-type mice are resistant to developing cardiovascular diseases compared to humans (Barter et al., 2003; Gistera et al., 2022). Thus, transgenic mouse models are crucial for understanding atherosclerosis (Gistera et al., 2022). There are several transgenic mouse

models used in atherosclerosis research, the LDL receptor knockout ($LDLR^{-/-}$) and the apolipoprotein E knockout ($apoE^{-/-}$) mouse models are the most common ones (Gistera et al., 2022). We will focus on the $apoE^{-/-}$ mouse model as it was used in this thesis.

1.2.3.1 $ApoE^{-/-}$ transgenic mouse model

ApoE is a protein involved in the lipid metabolism and transport (Mahley & Rall, 2019). ApoE proteins are important for cholesterol homeostasis, thus, disruption in apoE function results in hypercholesterolemia (Lee et al., 2017; Plump & Breslow, 1995). This mouse model was created by knocking out the apoE gene in E14TG2a embryonic stem cells that were then injected into C57BL/6J blastocysts, creating transgenic mice with inefficient plasma lipid clearance, resulting in hyperlipidemia which is a risk factor for atherosclerosis (Pendse et al., 2009; Taconic Biosciences, 2024).

There are several advantages to using $apoE^{-/-}$ mice in atherosclerosis research:

1) In $apoE^{-/-}$ mice, intervention of high fat diet (HFD) is not necessary, however, with HFD, they exhibit larger plaques (Plump et al., 1992; Reddick et al., 1994). Moreover, this makes them useful models for research looking at diet specific effects.
2) They also have a similar atherosclerosis pathophysiology to that of humans, making them physiologically relevant models (Nakashima et al., 1994).
3) Like $LDLR^{-/-}$ mice, they are also very well characterized, making them advantageous for research.

Like any animal model, the $apoE^{-/-}$ mice model also has its drawbacks:

1) Due to interspecies differences, they have different plasma cholesterol distributions than humans (Gistera et al., 2022).
2) The main responsibility of apoE gene is to clear the plasma cholesterol however, it has additional athero-protective properties (Getz & Reardon, 2009). Thus, atherosclerosis development in apoE$^{-/-}$ mouse model might not be solely due to the disruption in the lipid clearance mechanism (Lee et al., 2017).
3) Female apoE$^{-/-}$ mice have bigger atherosclerotic plaques than their male counterparts, which is the opposite of what is seen in humans (Smith et al., 2010). Thus, measuring the plaque size to study arsenic-induced atherosclerosis using apoE$^{-/-}$ mice might not be the most relevant animal model.

Overall, data translation from animals to humans is always a drawback of transgenic animal models nevertheless, the use of transgenic animals in atherosclerosis research is immensely helpful and much needed.

1.3 Arsenic-induced atherosclerosis

Epidemiological studies have linked the exposure of high levels of arsenic in drinking water to a myriad of cardiovascular diseases, especially to atherosclerosis (Wang et al., 2002). Literature shows that arsenic induced atherosclerosis remains to be a risk even after controlling for smoking, hypertension and diet (Nong et al., 2016).

Arsenic-induced atherosclerosis has been extensively studied on transgenic animal models, such as the well-established apoE$^{-/-}$ mice. Chronic arsenic exposure induces atherosclerosis in male apoE$^{-/-}$ mice even in the absence of HFD, showing significant changes both in plaque size and key plaque components (Lemaire et al., 2011; Makhani et al., 2018). The changes in the plaque composition revealed that chronic arsenic

exposure pushes the plaque phenotype to a more unstable state by increasing lipid levels while decreasing smooth muscle cell and collagen levels (Lemaire et al., 2011). Again, in the absence of HFD, arsenic was shown to induce atherosclerosis correlating with increasing dosage from 0 parts per billion (ppb) to 200 ppb in apoE$^{-/-}$ males, suggesting that there is no safe limit for arsenic-induced atherosclerosis (Makhani et al., 2018). Besides diet, exposure window is also very important in arsenic-induced atherosclerosis. Prenatal arsenic exposure increases lesion formation in the offspring of pregnant apoE$^{-/-}$ mice (Srivastava et al., 2007). In addition, early post-natal exposure to arsenic exacerbated atheroma development and enhanced lesion inflammation (Negro Silva et al., 2021; Srivastava et al., 2009). While the withdrawal of arsenic exposure hindered the lesion development, lesions did not regress, suggesting that removal of arsenic do not reverse the damage to the vessel walls (Srivastava et al., 2009).

Of note, arsenic induces atherosclerosis in another mouse model, the apoE$^{-/-}$ / LDLR$^{-/-}$ mouse. Here, 10 ppm arsenic increases the lesion size after 18 weeks of time (Bunderson et al., 2004). This indicates the arsenic-induced atherosclerosis is not specific to the apoE$^{-/-}$ mouse model.

1.3.1 Mechanisms involved in arsenic-induced atherosclerosis

Inflammation: Even though the mechanism by which arsenic induces atherosclerosis is still unknown, pro-atherogenic mechanisms such as continuous inflammation, monocyte infiltration and altered levels of cellular adhesion molecules have been associated with arsenic exposure (Balakumar & Kaur, 2009; Duan et al., 2022). Epidemiological data demonstrated that adhesion molecules, such as VCAM-1 and ICAM-1, were elevated in

the blood of populations exposed to moderate to elevated levels of arsenic in drinking water (Chen et al., 2007; Wu et al., 2012). It has also been shown that oxidized LDL is linked to c-reactive protein levels (CRP), which is a predictor of cardiovascular diseases and systemic inflammation, in an arsenic-exposed population in Bangladesh (Karim et al., 2013). Moreover, monocyte chemoattractant protein 1 (MCP1) is a cytokine that is involved in recruiting monocytes upon pro-inflammatory signals (Deshmane et al., 2009). Increased levels of MCP1 were detected in both the arsenic-exposed apoE$^{-/-}$ mouse model and human subjects (Lutgens et al., 2005; Srivastava et al., 2009; Wu et al., 2003).

ROS and oxidative stress: Literature suggests that arsenic results in elevated levels of ROS which induces oxidative stress (Barchowsky et al., 1999). Oxidative stress alters nitric oxide homeostasis in endothelial cells resulting in changes in vascular tone (Simeonova & Luster, 2004; Willerson & Ridker, 2004). The maintenance of vascular homeostasis is important to prevent endothelial dysfunction (Sandoo et al., 2010). In addition, oxidized LDLs are a hallmark of atherosclerosis, and they create ROS and other bioactive molecules, such as aldehydes (Esterbauer et al., 1991). Malondialdehyde (MDA) and 4-hydroxy-trans-2-nonenal (HNE) are the main products created by oxidized lipids (Esterbauer et al., 1991). Atherosclerotic lesions of arsenic-exposed mice had increased levels of the protein adducts of MDA and HNE and immunizing this protein adducts in mice impeded the formation of atherosclerotic lesions (George et al., 1998; States et al., 2009; Zhou et al., 2001).

Altered lipid homeostasis: Arsenic also disrupts lipid homeostasis by altering the lipid metabolism, which is also a known factor in atherosclerosis (Cheng et al., 2011). Both *in vitro* and *ex vivo* studies showed that environmentally significant levels of arsenic impede

Abca1 and sterol regulatory element binding protein 1 (*Srebp1c*) gene expression in macrophages, key genes for cholesterol metabolism (A. M. Padovani et al., 2010). A change in their expression results in altered cholesterol efflux, contributing to subsequent atherosclerosis development (A. M. Padovani et al., 2010).

All these studies highlight that arsenic effect many components in atherosclerosis development, such as inflammation, oxidative stress, and altered lipid metabolism, suggesting that mechanism of arsenic-induced atherosclerosis is complex and multifactorial.

1.3.2 Arsenic-induced atherosclerosis in the context of diet

HFD is a diet where more than 30% of its total energy intake is provided by fats and it is a known risk factor for many cardiovascular diseases, especially atherosclerosis (Nishina et al., 1993; Qiao et al., 2021; Zhang et al., 1994). Intake of elevated levels of fats results in an abundance of cholesterol in the circulation that results in a state of hyperlipidemia (Maulana & Ridwan, 2021). If not treated, hyperlipidemia leads to atherosclerotic plaque formation (Hill & Bordoni, 2024).

Literature shows that even though arsenic and HFD combination treatment do not change the plaque size in male apoE$^{-/-}$ mice, it changes the levels of key plaque constituents (Lemaire et al., 2011). Post arsenic and HFD treatment of 13 weeks, atherosclerotic lesions of male apoE$^{-/-}$ mice had more lipids but less smooth muscle cells and collagen fibers, demonstrating a less stable plaque phenotype (Lemaire et al., 2011).

In addition, HFD is a known contributor to oxidative stress, which is one of the mechanisms involved in atherosclerosis development (Sun et al., 2020). It stimulates the

ROS and results in a constant inflammatory state, providing a pro-atherogenic environment necessary for atherogenesis (Serra-Majem et al., 2019).

Moreover, there is also a close relationship between arsenic exposure and nutrition. One of the most common modes of exposure to arsenic is through ingestion of contaminated food and drinks, mainly water, rice, apple juice and wine (Lamas et al., 2023). The effect of arsenic can be lessened or exacerbated by diet. While diets such as western diets revolving around foods rich in fats induces atherosclerosis even without arsenic, mediterranean diets rich in antioxidants may protect against atherosclerosis (Gardener et al., 2014; Ghosh et al., 2015). Supplements such as selenium are also shown to be beneficial against arsenic-induced atherosclerosis (Krohn et al., 2016). Lentils high in selenium were found to be efficacious against arsenic-induced atherosclerosis in male apoE$^{-/-}$ mice (Krohn et al., 2016).

1.3.3 Arsenic-induced atherosclerosis in the context of sex

There are sexual dimorphisms in several pro-atherogenic mechanisms involving inflammation and oxidative stress (Martínez de Toda et al., 2023). Since oxidative stress and inflammation are also involved in arsenic induces atherosclerosis, there might be sex specific differences in development of arsenic induced atherosclerosis as well. The chronic inflammatory state of arsenic-induced atherosclerosis involves various immune cells and promotes ROS production, creating oxidative stress on the system (Batty et al., 2022). While in general, women generate ROS in lower quantities compared to their male counterparts, they also have more efficient antioxidant mechanisms to deal with the oxidative burden created by ROS (Borras et al., 2003). In contrast, men are worse at

managing oxidative stress via low antioxidant capacity, while producing higher levels of ROS and having a higher basal level of systemic inflammation due to a surplus of ROS (Borras et al., 2003; Martínez de Toda et al., 2023). *In vivo* murine studies demonstrate that males have inefficient mitochondrial electron transport chains, resulting in a leak of superoxide anions while producing ATP (Martínez de Toda et al., 2023). The leakage is the main site where ROS is generated, making men more vulnerable to oxidative stress which might contribute to arsenic induced atherosclerosis (Martínez de Toda et al., 2023).

Even though there are controversial findings in the literature, estrogen is linked to decreased levels of oxidative stress due to its scavenging properties (Arias-Loza et al., 2013). The phenolic hydroxyl group on the A ring of estrogen has shown to scavenge free radicals (Kadoma & Fujisawa, 2007). In addition, studies done on both humans and nonhuman primates show accelerated atherosclerosis progression in the absence of estrogen (Mikkola & Clarkson, 2002). All these findings indicate that female sex hormone is involved in atherosclerosis progression through oxidative stress management, which may suggest sex specific differences in arsenic induced atherosclerosis.

Overall, studies indicate that men have increased atherosclerotic burden and higher risk plaque features compared to women when they develop atherosclerosis (Man et al., 2020). However, it is still unknown how sex contributes to arsenic-induced atherosclerosis due to lack of research. An analysis involving 771 preclinical articles on cardiovascular diseases including atherosclerosis done by Ramirez and Hibbert revealed that 18.8% of studies did not specify the sex of the animals used in the studies and of the studies that did specify the sex, 20.4% studied females whereas 55.4% studied males alone. Thus, there is almost double the number of studies involving males. This analysis also

demonstrates that of the articles that mention the sex of the animals studied, less than 25% used both sexes to report sex as biological variable (Man et al., 2020; Ramirez & Hibbert, 2018). Of note, this analysis included studies using *in vivo* non-human mammals.

Females have been excluded from scientific studies throughout the history due to hormonal fluctuations and data variability. On the contrary, these excuses are the very reason investigators should include both sexes in their studies. When it comes to cardiovascular diseases, the world's leading cause of death, and arsenic contamination, one of the most significant chemical contaminants worldwide, inferring from male data is simply not enough to have a complete understanding on female cardiovascular health (World Health Organization, 2020a, 2020c).

1.4 Purpose of the thesis

Previously, our lab characterized the plaque size and constituents in male apoE$^{-/-}$ mice (Lemaire et al., 2011). Male mice were exposed to 200 ppb sodium arsenite for 13 weeks in the presence and absence of HFD to assess plaque size and constituents. The findings showed that there was an increase in plaque size post arsenic treatment but only in the group that was fed a PD. Additionally, this study identified arsenic-induced changes in plaque constituents that were similar in both diet groups (Lemaire et al., 2011). There were no significant changes in macrophage levels, but levels of lipids were increased whereas levels of smooth muscle cells and collagen fibers were decreased (Lemaire et al., 2011). However, how arsenic affects the female plaques remained unknown. Thus, we wanted to investigate the sex specific differences in arsenic-induced atherosclerosis in the context of diet, which is a known contributor to atherosclerosis. We used the same exposure parameters as in the study done on males and had the two following aims:

1.4.1 First aim

Our first goal was to characterize the size of atherosclerotic plaques in the aortic sinus of female apoE$^{-/-}$ mice in the context of diet.

1.4.2 Second aim

Our second goal was to assess the levels of different plaque constituents, namely, lipids, collagen fibers, macrophages, and smooth muscles cells.

The findings of these aims have been written as a manuscript presented in the second chapter of this thesis.

Chapter 2: Characterization of Atherosclerotic Plaque in Female Apolipoprotein E Knockout Mice Post Arsenic Exposure

This study is in preparation for publication:

Zengin A. N., Gagnon N., Subramaniam N., Mann K.K. Characterization of Atherosclerotic Plaque in Female Apolipoprotein E Knockout Mice Following Arsenic Exposure

Authors contribution:

Ayse Nazli Zengin: performed experiments, analyzed the data, and wrote the manuscript.

Natascha Gagnon: performed experiments.

Nivetha K. Subramaniam: performed experiments.

Koren K. Mann: conceptualized experiments, edited the manuscript, and provided funding.

Acknowledgement:

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2.1 Abstract

Arsenic exposure is a significant public health issue worldwide, endangering millions through contaminated food, air, and water. Exposed populations face serious long term health issues such as vascular diseases like atherosclerosis. In male mice, chronic arsenic exposure increases the size of atherosclerotic plaques and alters plaque composition however, the impact on female mice has yet to be explored. In this study, we aimed to discover how female mice were affected by chronic arsenic exposure under different dietary conditions. Female apolipoprotein E knockout ($apoE^{-/-}$) mice were exposed to 200 parts per billion (ppb) sodium arsenite or tap water with or without high fat diet (HFD) for 13 weeks. Plaque size and plaque composition in the aortic sinus were characterized. While arsenic did not increase plaque size in female mice, there was an increase in lipid content within the atherosclerotic lesions. These data contrasted with historical male data, thus demonstrating sex-specific effects of arsenic on atherosclerotic plaque development.

Keywords: Arsenic, atherosclerosis, sexual dimorphism, high fat diet, plaque size and composition

2.2 Introduction

Arsenic, a metalloid naturally found in the environment, is a globally recognized contaminant affecting millions worldwide (Chung et al., 2014; Kuivenhoven & Mason, 2023). Although arsenic exposure can occur through different routes of exposure, the

most common route is ingestion, either through contaminated groundwater, often used as irrigation and drinking water, or contaminated food (Agency for Toxic Substances and Disease Registry, 2007; Malakar et al., 2019). According to the World Health Organization (WHO), the top 10 countries with contaminated groundwater with naturally elevated levels of arsenic include China, India, the United States of America (USA), Pakistan, and Bangladesh, which are also the places with the highest population in the world (World Health Organization, 2022). Thus, millions of people are exposed to high arsenic levels. In addition, high arsenic exposure can also be due to anthropogenic activities, including use of fossil fuels (Dai et al., 2005; Liu et al., 2017), agricultural use of pesticides (Islam et al., 2016), smelting (Schlesinger et al., 2022) and mining (Garelick et al., 2009; Smith et al., 2003).

Arsenic exposure causes skin diseases (Choudhury et al., 2018; Dastgiri et al., 2010), neurological disorders (Jiang et al., 2014; Mukherjee, 2003), various cancers (Marshall et al., 2007; Wong & Wang, 2010), and metabolic (Guha Mazumder, 2001; Lai, 1994; Navas-Acien, 2008) and cardiovascular diseases, such as atherosclerosis (Medrano, 2010; Moon et al., 2013). Atherosclerosis is a cardiovascular disease characterized by chronic inflammation and fibro-fatty build up in the arteries, leading to arterial thickening and hardening (Lichtenstein, 2003). This fibrous build up is often referred to as a plaque or an atheroma. During atherogenesis, oxidized low density lipoproteins activate endothelial cells causing expression of adhesion molecules that recruit monocytes which transmigrate through the endothelium to the intima (Chen & Khismatullin, 2015). Here, they differentiate into proatherogenic macrophages which retain the lipids and form foam

cells (Poznyak et al., 2021). As the plaques grow, smooth muscle cells migrate towards the endothelium and secrete a collagen cap to stabilize the developing plaques (Harman & Jørgensen, 2019). Foam cells undergo apoptosis, resulting in a necrotic core, destabilizing the plaques (Yuzhou Gui et al., 2022). Unstable plaques then may dislodge and cause thrombosis, which can eventually result in stroke and myocardial infarction (Bentzon et al., 2014b).

While there are many risk factors for atherosclerosis including smoking, sedentary lifestyle, hypertension, obesity, and unhealthy diets containing high cholesterol, the American Heart Association issued a statement stating that metals, including arsenic, are associated with the development of atherosclerosis (Lamas et al., 2023). Multiple epidemiologic studies have identified a link between arsenic and atherosclerosis in human populations. A longitudinal cohort study done in Spain linked heavy metals including arsenic to atherosclerosis particularly in the carotid arteries (Grau-Perez et al., 2022). Likewise, a dose-response meta-analysis study showed a 94% increased risk for carotid atherosclerotic disease upon chronic arsenic exposure as low as the WHO guideline limit of 10 ppb, affecting a significant global population compared to a reference population exposed to 1 ppb. (Xu et al., 2020).

Arsenic-induced atherosclerosis has been extensively modeled in mice, including the well-characterized apolipoprotein E knock out mouse ($apoE^{-/-}$) model. Chronic arsenic exposure aggravates inflammation and atheroma formation both post-natally and after an

early-life exposure (Lemaire et al., 2011; Negro Silva et al., 2021; Srivastava et al., 2009). Of note, arsenic induces atherosclerosis in male apoE$^{-/-}$ mice even without a high fat diet (Lemaire et al., 2011; Makhani et al., 2018). Arsenic increased atherosclerosis in a dose dependent manner in male apoE$^{-/-}$ mice up to 200 parts per billion (ppb), when mice were fed a normal purified diet (PD) (Makhani et al., 2018). However, arsenic did not increase the plaque size when mice were fed a high fat diet (HFD), although there were alterations in plaque constituents independent of diet (Lemaire et al., 2011). Arsenic decreased smooth muscle cells and collagen fibers, while increasing lipids in male apoE$^{-/-}$ mice (Lemaire et al., 2011). Macrophage staining remained unchanged by arsenic exposure (Lemaire et al., 2011).

All the previous studies of post-natal arsenic exposure have utilized male mice. Women are protected from cardiovascular disease when compared to men, at least prior to menopause (Man et al., 2020). However, female apoE$^{-/-}$ mice have elevated plaque levels compared to males (Man et al., 2020; Matsumoto et al., 2016; Zhang et al., 2018). It is unknown whether there is a sexually dimorphic response to arsenic with regards to atherosclerosis. Therefore, we investigated how moderate levels of arsenic affected atherosclerosis development in female apoE$^{-/-}$ mice. Interestingly, our data suggest that females are protected against arsenic-induced atherosclerosis compared to males.

2.3 Materials and methods

Animal housing and arsenic exposure: This study was approved by and conducted in accordance with the regulations of the McGill Animal Care and Use Committee (AUP JGH-5664). Apolipoprotein E knockout (B6.129P2-ApoE^{tm1Unc}/J; apoE$^{-/-}$) mice were purchased from the Jackson Laboratory and bred at the Lady Davis Institute. Four-week-old female apoE$^{-/-}$ mice were fed either the purified diet (AIN-76A diet; Envigo, QC, Canada) or high fat diet (2016 + 15% Cocoa Butter + 0.5% Chol. (TD.10825); Envigo, QC, Canada). Purified murine chow was used to limit metal exposure through diet (Murko et al., 2018). At 5-weeks of age, animals from both diet groups were randomly separated into two groups and were exposed to either tap water or 200 ppb arsenic. A sample size of 8 was utilized in each group. Arsenic water was prepared by dissolving sodium arsenite (0.35 mg/L $NaAsO_2$ (S7400-100G); Sigma-Aldrich, ON, Canada) in tap water. Water bottles were changed 3 times a week for both control and arsenic cages to ensure each cage was treated equally and to limit arsenic oxidation. After 13 weeks, animals were sacrificed using isoflurane followed by a cardiac puncture. The blood from the cardiac puncture was kept for blood plasma analyses, whereas hearts and aortae were dissected and fixed in formalin for 24 hours (Figure 1A).

Plasma analyses: Blood was collected from cardiac puncture and plasma was separated utilizing ethylenediaminetetraacetic acid containing collection tubes [K2 EDTA 7.2mg (367861); BD vacutainer, Franklin Lakes, NJ, USA]. Plasma was flash frozen upon separation and was sent to The Center of Phenogenomics (Toronto, Ontario) for liver function tests consisting of alanine transaminase (ALT) and aspartate transaminase (AST) assessment.

Characterization of atherosclerotic plaques: Plaque characterization was conducted as previously described (Lemaire 2011; Makhani 2018). Briefly, the hearts were rinsed, fixed, embedded and processed. Each slice was 7 μm thick and collected on 10 different slides. Each slide, containing 5 to 10 slices, represented the whole sinus. Slides were stained with oil red O (oil red O solution (26609-01); Electron Microscopy Sciences, Hatfield, PA, USA) and then imaged with INFINITY CAPTURE software and camera (INFINITY CAPTURE; Lumenera, ON, Canada) at two magnifications. Lower (5x) magnification images were used to assess plaque size, whereas 10x magnification images were used to assess percent lipid. Percent collagen in aortic sinus was assessed using picrosirius red stain kit [picrosirius red stain solution A (24901A), picrosirius red F3BA stain solution B (24901B), and .01N hydrochloride acid solution C (24901C); Polysciences, Warrington, PA, USA]. After the stain, the slides were imaged under polarized light.

Immunofluorescent staining: Macrophage and smooth muscle cell content of the aortic sinuses were assessed as previously mentioned (Lemaire et al., 2011; Makhani et al., 2018). In short, sinuses were blocked with 3% bovine serum albumin for an hour and stained with 1:50 diluted anti-MOMA-2 (anti-MOMA-2 (ab33451), Abcam Inc., Toronto, ON, Canada) or 1:100 diluted anti-α-smooth muscle actin (anti-α-smooth muscle actin (ab7817-500), Abcam Inc., Toronto, ON, Canada). Followed by a PBS wash (D-PBS 1X (311-425-CL); Wisent Inc., Saint-Jean-Baptiste, QC, Canada), 1:500 diluted goat anti-rat conjugated with Alexa 488 (Goat anti-Rat AF488 (A-11006); Life Technologies Corporation, Eugene, OR, USA) and 1:500 diluted goat anti mouse conjugated with Alexa

488 (Goat anti-Mouse AF488 (A-11029); Life Technologies Corporation, Eugene, OR, USA) were used as secondary antibodies, respectively. After an hour incubation, slides were washed with PBS and deionized water and stained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride (D8417), Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes. Samples were mounted with Immu-Mount [Immu-Mount (9990402); Epredia, Kalamazoo, MI, USA] and imaged with INFINITY CAPTURE software and camera within a week of stain.

Statistical analysis: For statistical analysis, an independent t test was performed to compare the arsenic-treated group to the control group using GraphPad Prism 9.5.0 software. Statistical significance was indicated by a p value < 0.05 and the data are represented as the mean with standard deviation.

2.4 Results

Arsenic exposure did not change the plaque size in female apoE$^{-/-}$ mice in either diet group.

Previously, our lab exposed male apoE$^{-/-}$ mice to 200 ppb arsenic and assessed plaque size and constituents (Lemaire et al, 2011). These levels of arsenic exposure in drinking water increased plaque size and altered plaque constituents into a less stable plaque phenotype in male apoE$^{-/-}$ mice. Here, we investigated how arsenic affected the plaque size in female apoE$^{-/-}$ mice exposed to either tap water or 200 ppb arsenic for 13 weeks

(Figure 1A). The size of atherosclerotic plaques in the aortic sinus was assessed with oil red O staining. The mean plaque size was larger in the HFD-fed animals than their PD-fed counterparts (Figure 1B-C), which is well established in the literature (Smith et al., 2010). However, arsenic treatment had no impact on plaque size in the aortic sinuses of either diet group (Figure 1B-C). This contrasts with data from male mice showing that arsenic increases plaque size (Lemaire et al., 2011; Srivastava et al., 2009).

Arsenic exposure increased the lipid levels of atherosclerotic lesions in female apoE$^{-/-}$ mice in the absence of high fat diet.

Arsenic increases the macrophage intracellular cholesterol levels by inhibiting the lipid efflux system, resulting in an imbalance of macrophage cholesterol homeostasis (A. M. S. Padovani et al., 2010). Thus, arsenic exposure correlated with in increased lipid retention within the plaques of male apoE$^{-/-}$ mice but does not change the percent of macrophage staining (Lemaire et al., 2011; A. M. S. Padovani et al., 2010). Here, we assessed the lipid and macrophage levels in atherosclerotic lesions in the aortic sinus of female apoE$^{-/-}$ mice using oil red O and MOMA-2 staining, respectively. Post arsenic treatment, lipid levels were significantly increased ($p \leq 0.001$) in the PD-fed group, while the macrophage levels remained unchanged (Figure 2A & C). Surprisingly, there was no change in lipid or macrophage levels of the HFD group (Figure 2B & D).

Arsenic exposure did not alter the levels of smooth muscle cells or collagen fibers of atherosclerotic lesions in female apoE$^{-/-}$ mice.

We also determined smooth muscle cell and collagen levels in atherosclerotic lesions through staining using anti-α-SMA and picrosirius red, respectively. In atherosclerosis, plaques have fibrous caps made of connective tissue, smooth muscle cells and collagen, protecting them from rupture. Once the fibrous cap is eroded, the plaque is less stable, and the blood flow may result in plaque dislodgement and subsequent thrombosis. Previously, in male apoE$^{-/-}$ mice, we showed that arsenic leads to unstable atherosclerotic plaques by reducing the levels of smooth muscle cells in atherosclerotic lesions and subsequent collagen production the by smooth muscle cells (Lemaire et al., 2011). In contrast, there were no significant differences in collagen or smooth muscle cell staining in either diet group post arsenic treatment in females (Figure 3 A-D).

Arsenic exposure increased AST levels in female apoE$^{-/-}$ mice in the presence of HFD.

In order to control for potential systemic toxicities, we assessed liver function by plasma AST and ALT levels. There was no meaningful change in ALT levels in either diet group (Figure 4 A-B), however after arsenic treatment, AST levels of high fat diet females were significantly increased (Figure 4D), indicating there might be tissue damage induced by arsenic and high fat diet combination, although this did not correlate with increased atherosclerosis.

2.5 Discussion

Epidemiological data and a recently issued AHA Scientific Statement connect metals, including arsenic, to an elevated risk of cardiovascular diseases, such as acute myocardial infarction, systemic hypertension, and atherosclerosis (Chen & et al., 1995; Grau-Perez et al., 2022; Lamas et al., 2023; Monrad et al., 2017). Likewise, animal data show that chronic arsenic exposure increases the development of atherosclerotic lesions and alters plaque composition in *in vivo* mouse models (Lemaire et al., 2011; Makhani et al., 2018). Early-life and postnatal arsenic exposure causes significant increase in atheroma formation in apoE$^{-/-}$ mice, a very well-established animal model in atherosclerosis research (Negro Silva et al., 2021). However, atherosclerotic lesion formation following post-natal arsenic exposure has not been characterized in female mice. Herein, we describe our findings that females are not as sensitive to arsenic-induced atherosclerosis as males. There were no changes in plaque size, and only minimal changes in plaque constituents following exposure to arsenic where only lipid levels were elevated post arsenic exposure in the absence of high fat diet. These data contrast with data from male apoE$^{-/-}$ mice, where 200 ppb arsenic increased plaque size but also altered plaque composition (Lemaire et al., 2011). Females did exhibit increased AST when concomitantly exposed to HFD and arsenic, while males did not (Lemaire et al., 2011). However, like males, the percentage of lipid staining within the plaque was increased by arsenic (Lemaire et al., 2011).

Wild-type rodents do not develop atherosclerosis thus, transgenic mice are utilized as models to study atherosclerosis (Gistera et al., 2022; Zaragoza et al., 2011). In the

apoE$^{-/-}$ transgenic mouse model, the clearance of the blood cholesterol is compromised, and the animals are rendered hyperlipidemic, promoting the spontaenous development of atherosclerosis even without the supplement of high fat diet (Ishibashi et al., 1994; Lemaire et al., 2011; Pendse et al., 2009). This allows researchers to observe disease progression from early onset to later stages of atherosclerosis, as the pathophysiology of atherosclerosis is remarkably like that of humans (Man et al., 2020; Nakashima et al., 1994). ApoE$^{-/-}$ mice develop atherosclerosis faster with the addition of high fat diet, analogous to humans (King et al., 2010).

However, it is well known that there are differences by sex in the development of atherosclerosis in both mice and humans (Man et al., 2020). Unlike humans, male apoE$^{-/-}$ mice have smaller plaques than female apoE$^{-/-}$ mice, although there is some controversy as to whether this persists in aged mice (Man et al., 2020; Matsumoto et al., 2016; Zhang et al., 2018). Spontaneous plaque rupture does not occur in mice, however, various surrogate outcomes such as necrotic core size, lipid percentage and inflammation are used to overcome this limitation, making characterization of plaque constituents important (Man et al., 2020). In contrast, the inflammatory component of plaque in male apoE$^{-/-}$ mice is greater and more similar in composition to humans (Man et al., 2020).

Multiple pro-atherogenic mechanisms exhibit differences between sexes. The chronic inflammatory nature of atherosclerosis involves many immune cells, but also the generation of reaction oxygen species (ROS). Women produce less ROS and have more

efficient antioxidant mechanisms to deal with the oxidative stress created by ROS (Bhatia et al., 2012; Matarrese et al., 2011). Men have higher basal inflammation, but dampened antioxidant mechanisms, resulting in a heavier oxidative burden (Bhatia et al., 2012; Matarrese et al., 2011). Arsenic induces ROS in the apoE$^{-/-}$ vasculature of male mice (Lemaire et al., 2015; Matarrese et al., 2011), thus females are perhaps protected from arsenic-induced atherosclerosis based on their increased antioxidant response system. Males have less efficient mitochondrial electron transport systems, resulting in superoxide anion leak that promotes more ROS production than their female counterparts (Toda & et al., 2023). The antioxidant enzymes superoxide dismutase and glutathione peroxidase are less active and less expressed in males than females, resulting in excess ROS to accumulate and subsequent oxidative stress (Toda & et al., 2023). Moreover, estrogen is a modulator of oxidative stress, where higher estrogen levels decrease oxidative stress by impeding ROS production and acting as a free radical scavenger (Stepniak & Karbownik-Lewinska, 2016). It is possible that women catch up to their male counterparts after menopause in disease burden, due to plummeting estrogen levels and following decreased efficiency of antioxidant mechanisms.

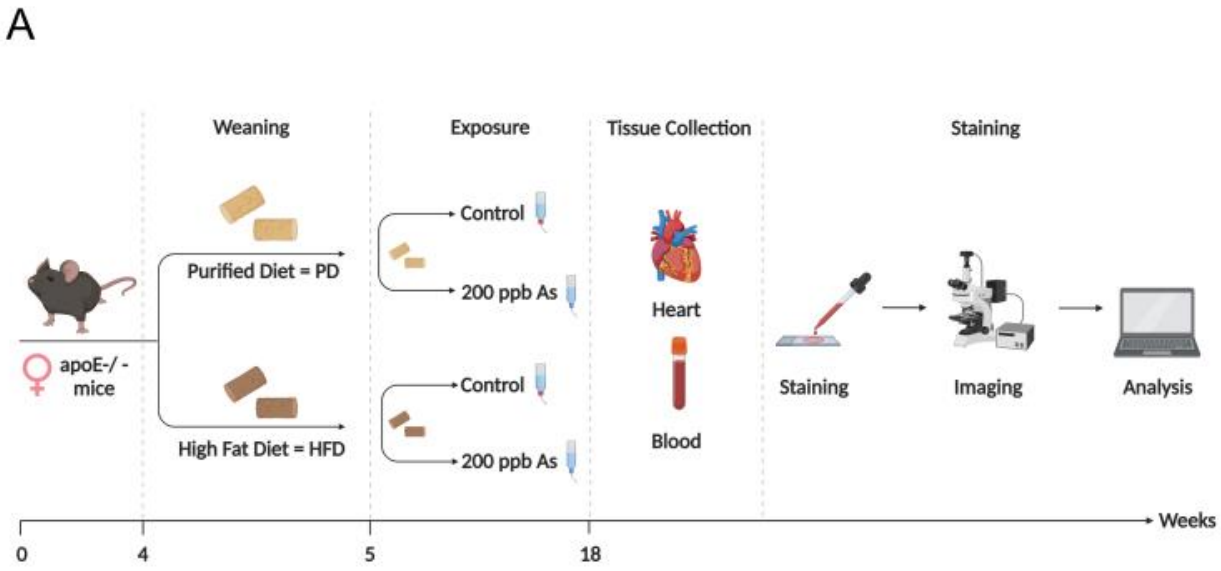
The rate of biotransformation of arsenic is also different between sexes. The enzyme arsenic 3 methyltransferase (As3MT) successively methylates arsenic to monomethylated, demethylated, and trimethylated arsenic metabolites (Stýblo et al., 2021). We have previously demonstrated that deletion of As3MT protects male apoE$^{-/-}$ mice from arsenic-induced atherosclerosis (Negro Silva et al., 2017). This suggests that arsenic methylation, or potentially the rate thereof, is an important parameter when

considering atherosclerosis outcomes. Females methylate faster than males (Muhetaer et al., 2022; Shen et al., 2016) and perhaps increased clearance of toxic methylated metabolites decreases the pro-atherogenic response. Thus, the rate of arsenic biotransformation might be an important predictor of the pro-atherogenic effects of arsenic. In human populations, the methylation index (a ratio of mono-methylated to inorganic arsenic) correlates with cardiovascular disease, suggesting this may be true (Kuo et al., 2022). This question can be addressed by utilizing the humanized As3MT mice recently described (Douillet et al., 2023). Mice are much more efficient at metabolizing arsenic than humans (Vahter, 1999). thus apoE$^{-/-}$ mice that carry the slower-methylating human As3MT would be predicted to exhibit greater arsenic-induced atherosclerosis. One might predict that male humanized As3MT/apoE$^{-/-}$ mice would be exhibit greater arsenic-induced plaque than females.

In this study, we exposed female apoE$^{-/-}$ mice to 200 ppb arsenic in order to compare with our previous studies in male animal (Lemaire et al., 2011). Of note, this is 20 times the WHO allowable limit of arsenic for drinking water. However, due to the efficient nature of arsenic metabolism in mice, this exposure approximately equals to humans exposed to 40 ppb arsenic, according to the calculations of human equivalent dose reported by U.S. Food and Drug Administration (FDA, 2005; Negro Silva et al., 2021). In Bangladesh, it is estimated that 27 million people are exposed 50 ppb arsenic through drinking water, demonstrating that the dose used in this study is relevant to human exposure in places with naturally high arsenic levels (Ravenscroft P et al., 2009.; Yunus et al., 2016). Importantly, our lab has shown that arsenic concentrations as low as 10 ppb has enhanced atherosclerosis in male apoE$^{-/-}$ mice (Makhani et al., 2018).

Overall, the work discussed here shows that females are affected by arsenic treatment to a lesser extent than males. This underlines the importance of assessing sex as a biological variable in toxicology and cardiovascular research. The lack of consideration of both sexes produces challenges in comparing data and creates knowledge gaps in discovering sex specific mechanisms. Extrapolation of findings derived from only one sex can impair defining at-risk populations or development of therapeutic interventions. Beyond sex, age and hormone-status are also particularly important variables to consider in arsenic-induced atherosclerosis. After menopause, women have similar cardiovascular risks as men thus, modulating hormones or investigating atherosclerosis in aged mice of both sexes are important future experiments.

2.6 Figures


A
Weaning
Exposure
Tissue Collection
Staining
Purified Diet = PD
High Fat Diet = HFD
apoE-/- mice
Control
200 ppb As
Control
200 ppb As
Heart
Blood
Staining
Imaging
Analysis
Weeks
0
4
5
18

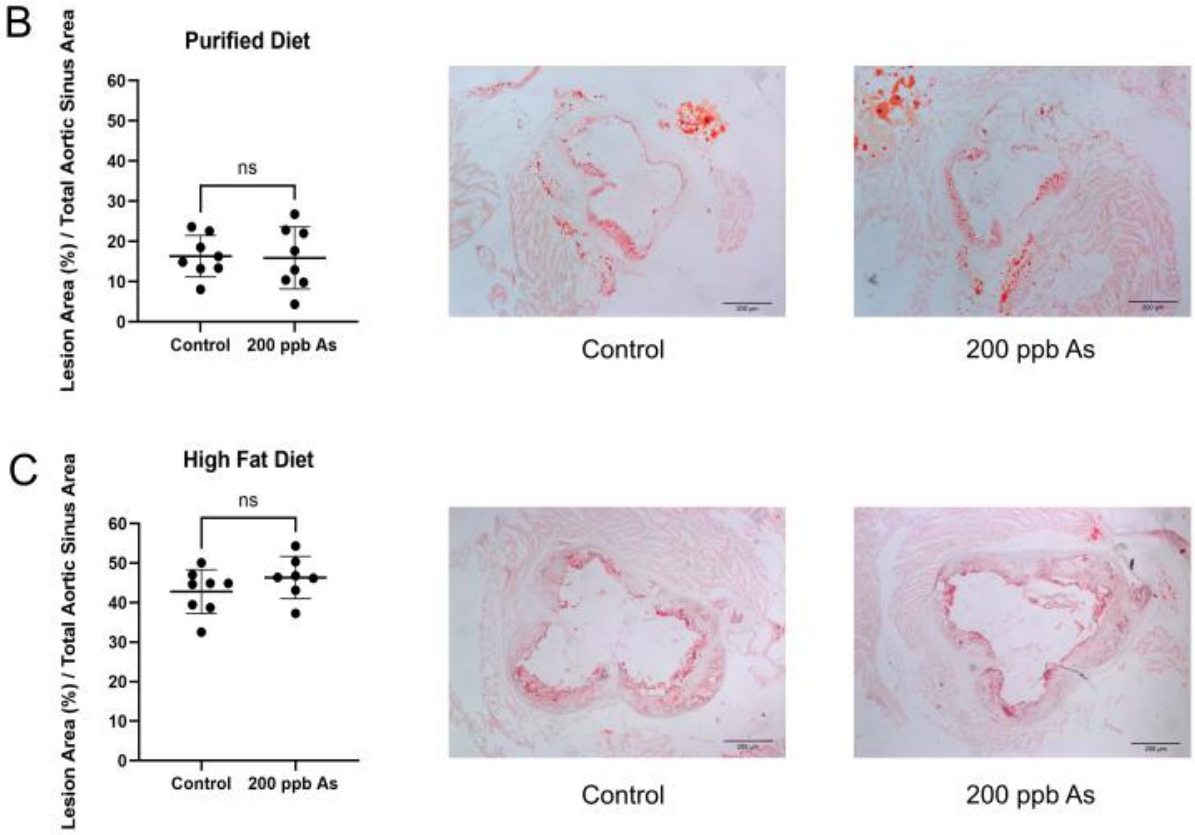

B
Purified Diet
Lesion Area (%) / Total Aortic Sinus Area
ns
Control
200 ppb As
Control
200 ppb As
C
High Fat Diet
Lesion Area (%) / Total Aortic Sinus Area
ns
Control
200 ppb As
Control
200 ppb As

Figure 1: Plaque size in aortic sinus did not change with arsenic exposure in female apoE$^{-/-}$ mice. Female apoE$^{-/-}$ mice were exposed to 200 ppb or tap water and given either PD or HFD for 13 weeks. Cryosectioned heart samples were stained with oil red O and analyzed using ImageJ (A). PD fed mice had smaller plaques than HFD fed mice (B and C, respectively), although arsenic did not significantly change plaque size. Values are shown as mean $\pm$ SD. (ns = $p > 0.05$, $n = 8$) along with representative images.

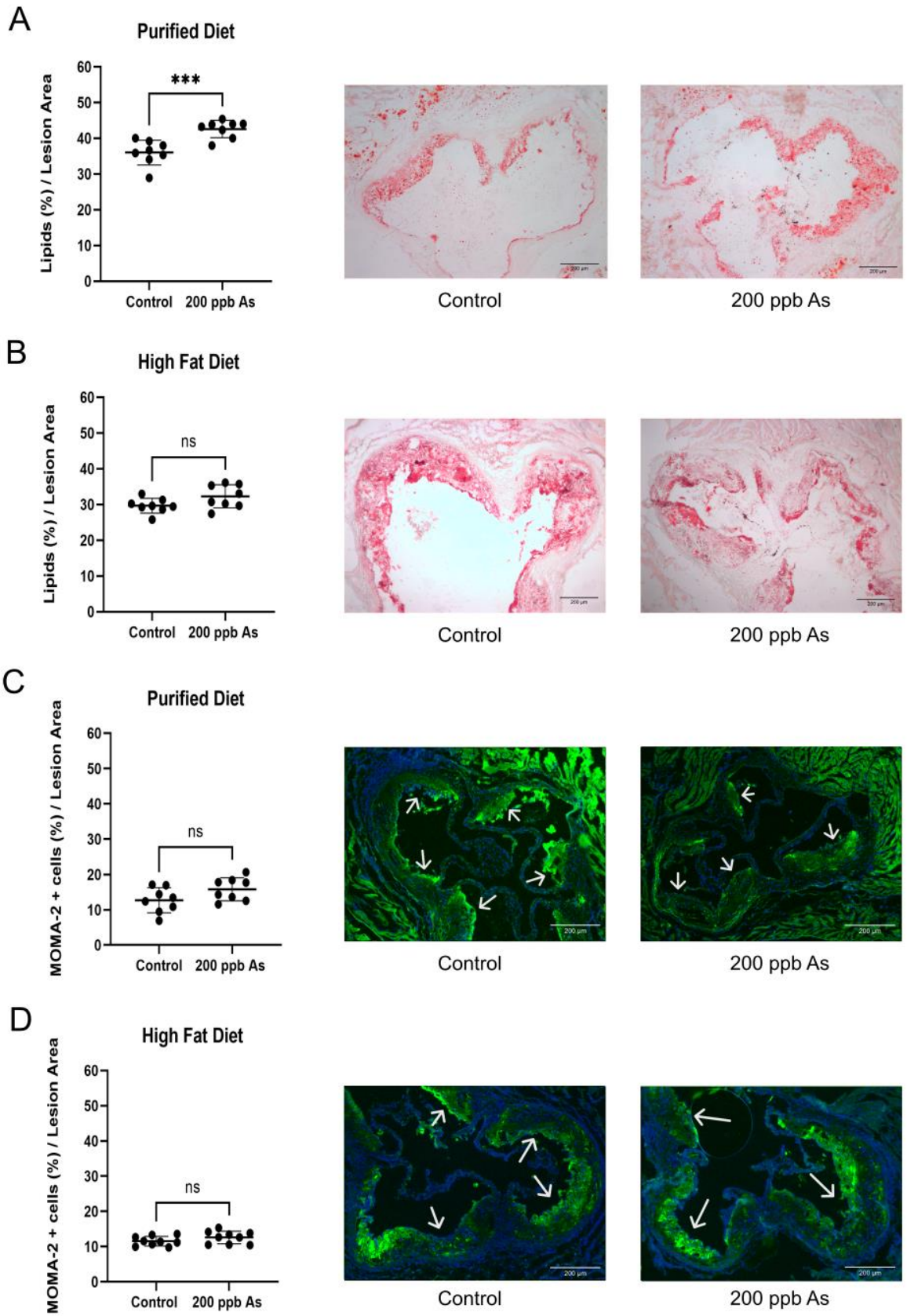
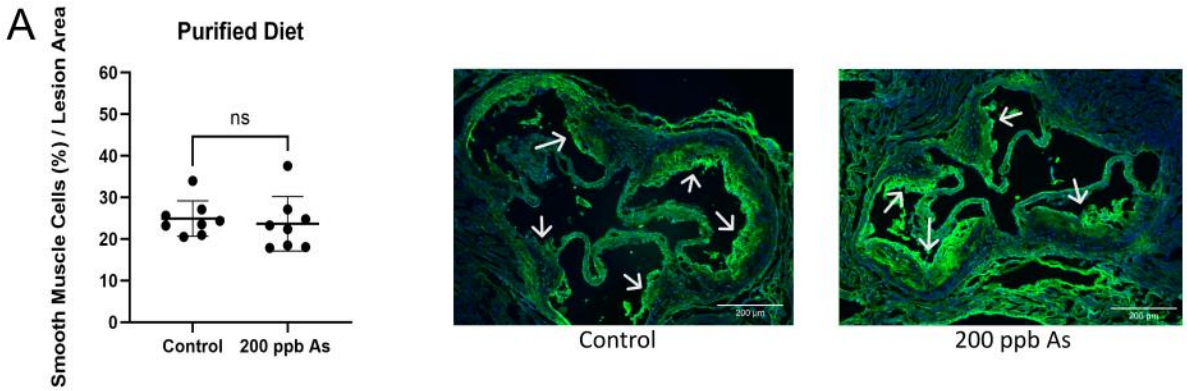
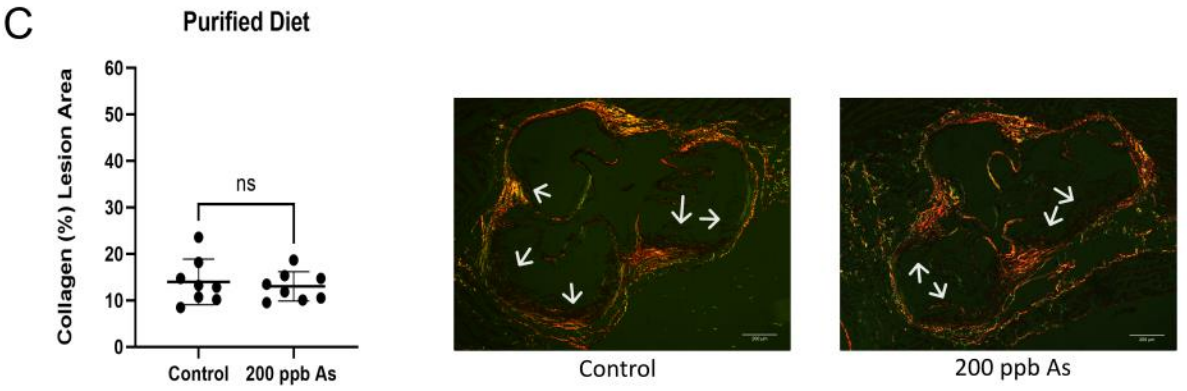

A
Purified Diet
Lipids (%) / Lesion Area
***
Control
200 ppb As
Control
200 ppb As
B
High Fat Diet
Lipids (%) / Lesion Area
ns
Control
200 ppb As
Control
200 ppb As
C
Purified Diet
MOMA-2 + cells (%) / Lesion Area
ns
Control
200 ppb As
Control
200 ppb As
D
High Fat Diet
MOMA-2 + cells (%) / Lesion Area
ns
Control
200 ppb As
Control
200 ppb As

Figure 2: Chronic arsenic exposure induces an increase in lipid content of atherosclerotic plaques in the context of PD. Post arsenic exposure, lipid levels of atherosclerotic lesions were increased only in the absence of HFD (A and B). Moreover, macrophage levels in either diet group were not altered by the arsenic exposure (C and D). Plaques were stained with oil red O and MOMA-2 for lipid and macrophage assessment, respectively and representative images are shown. (ns = $p > 0.05$, *** = $p \leq 0.001$, mean $\pm$ SD, n = 8)


A
Purified Diet
Smooth Muscle Cells (%) / Lesion Area
60
50
40
30
20
10
0
ns
Control
200 ppb As
Control
200 ppb As


B
High Fat Diet
Smooth Muscle Cells (%) / Lesion Area
60
50
40
30
20
10
0
ns
Control
200 ppb As
Control
200 ppb As


C
Purified Diet
Collagen (%) Lesion Area
60
50
40
30
20
10
0
ns
Control
200 ppb As
Control
200 ppb As

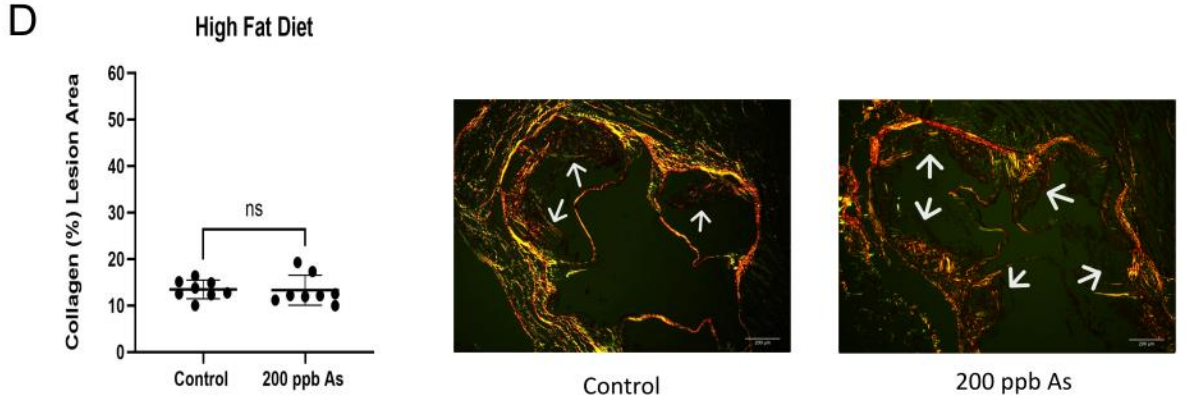

D
High Fat Diet
Collagen (%) Lesion Area
60
50
40
30
20
10
0
ns
Control
200 ppb As
Control
200 ppb As

Figure 3: Levels of smooth muscle cells and collagen fibers within atherosclerotic lesions remained unchanged post chronic arsenic exposure. Neither treatment nor diet altered the percent smooth muscle cells (A and B) or collagen fibers in the plaques (C and D). All plaques were stained with anti-SMC antibody and picrosirius red for the assessment of smooth muscle cells and collagen fibers, respectively. Images were analyzed using Image J. Unpaired T test was performed using Graphpad Prism. (ns = $p > 0.05$, mean $\pm$ SD, n = 8)

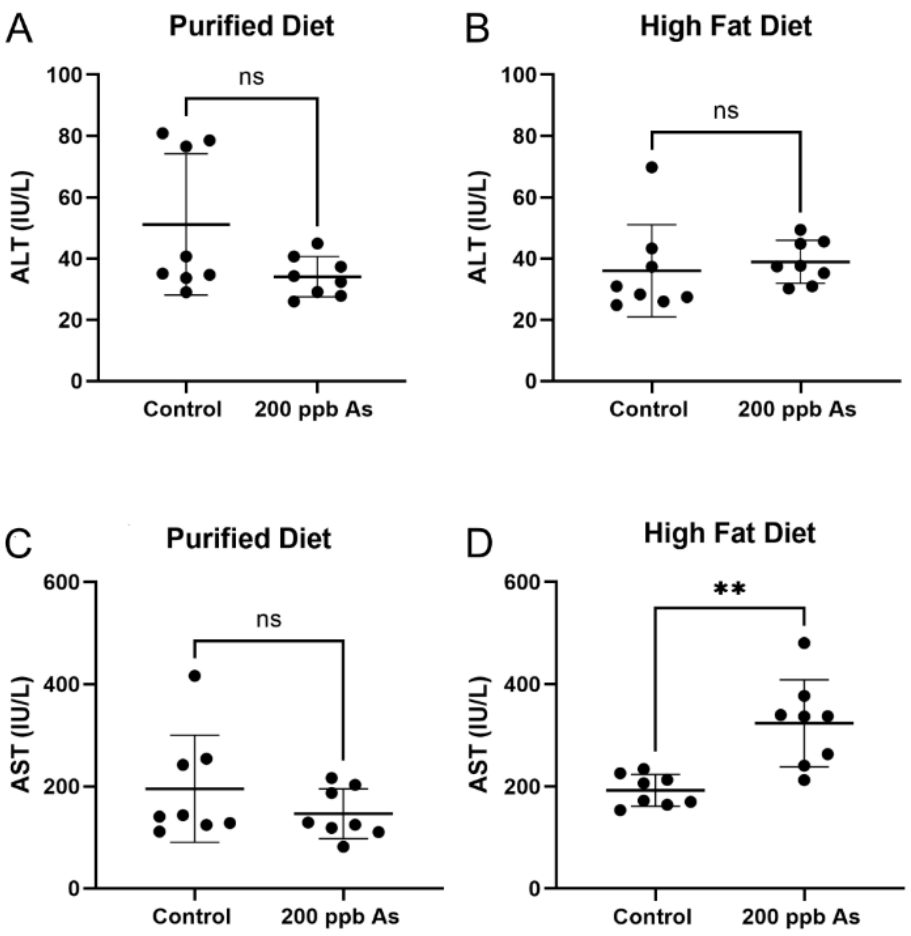


Figure 4: Post chronic arsenic treatment, ALT levels remained unchanged in either diet group however AST levels were elevated in the HFD group only. Plasma was collected from the cardiac blood of apoE$^{-/-}$ mice and liver enzymes analyzed. Unpaired T test was performed. (ns = $p > 0.05$, ** = $p \leq 0.01$, mean $\pm$ SD, n = 8)

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CRediT Authorship Contribution statement:

Ayse Nazli Zengin: Conceptualization, Investigation, Formal Analysis, Project Administration, Writing - original draft, Writing - review & editing. **Natascha Gagnon:** Investigation. **Nivetha K. Subramaniam:** Investigation. **Koren K. Mann:** Conceptualization, Funding Acquisition, Writing - Review & Editing.

Declaration of Competing Interests:

Authors declare that there were no competing financial interests or personal relationships influencing the work mentioned above.

Data availability:

Upon request data will be available.

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Chapter 3: Overall discussion, summary and future avenues

3.1 Conclusions

Cardiovascular diseases, namely coronary artery disease and stroke, are the top two leading causes of death worldwide (World Health Organization, 2020c). However, like many diseases, cardiovascular diseases are subjected to sex specific differences causing them to differ in prevalence, symptoms and outcomes between men and women (Betai et al., 2024). Either alone or in combination, genetic, hormonal, and behavioral factors can lead to these differences. Understanding the etiology, pathophysiology, symptoms, and pathways involved in these diseases in both sexes are crucial for mitigation and treatment options.

Moreover, countless epidemiological data showed a link between cardiovascular diseases and environmental risk factors such heavy metals (Chen et al., 2006; Harari et al., 2019; Lamas et al., 2023). In fact, several organizations such as ASTDR, WHO and AHA, mentioned arsenic as a risk factor for cardiovascular diseases, specifically atherosclerosis and set limits for public health safety (Agency for Toxic Substances and Disease Registry, 2007; Lamas et al., 2023; World Health Organization, 2022). Furthermore, exposure windows and combination with different diet types are shown to be crucial for arsenic induced atherosclerosis development. Negro Silva et al. demonstrated that exposure to arsenic early in life or postnatally causes increased the levels of atherosclerotic plaque development in mice (Negro Silva et al., 2021). In addition, Lemaire et al. demonstrated that chronic exposure to moderate levels of arsenic results in increased plaque formation and altered plaque composition in male apoE$^{-/-}$ mice in the context of both HFD and PD while Makhani et al, showed a dose response

relationship between arsenic exposure and atherosclerosis development in doses as little as the allowable limit in drinking water set by WHO (Lemaire et al., 2011; Makhani et al., 2018).

Despite the known association between arsenic and atherosclerosis, and cardiovascular diseases and sexual dimorphisms, post-natal arsenic exposure-induced atherosclerotic plaque characterization in females has not been assessed. Thus, this project aimed to characterize the role of sex and diet in arsenic-induced atherosclerotic plaque development in female apoE$^{-/-}$ mice by replicating the investigation done on males previously by Lemaire et al.. We exposed 4-week-old female apoE$^{-/-}$ mice to PD or HFD for a week before starting them on either tap water or 200 ppb sodium arsenite for 13 weeks. At the age of 18 weeks, we collected the hearts and blood. The hearts were stained for the characterization of plaque size and constituents while the plasma was analyzed for enzymes assessing for liver function. The findings of this study provide compelling evidence that arsenic impacts females to a lesser extent than males, shedding light on sex specific differences in the development of arsenic induced atherosclerosis. While there were no changes in plaque size in either diet group, there were minimal changes in the plaque constituents in PD. For plaque size assessment, Lemaire et al. showed a significant increase in the PD fed males while no changes were observed in HFD fed males post arsenic exposure (Lemaire et al., 2011). Contrastingly, our results demonstrated that females had no changes in either diet group post arsenic exposure.

For the assessment of plaque constituents, Lemaire et al. demonstrated that after arsenic treatment while there were no changes in macrophage levels, there was a significant increase in lipid levels and a significant decrease in the levels of both smooth muscle

cells and collagen fibers (Lemaire et al., 2011). Moreover, these changes were uniform across both diets suggesting that observed changes were independent of diet treatment (Lemaire et al., 2011). Contrastingly, females had no changes in the levels of macrophages, smooth muscle cells and collagen fibers in either diet groups. However, there was a significant increase in the lipid levels only in the PD-fed females matching the male data.

These data clearly show that there are sex specific differences in the atherosclerotic plaque development in apoE$^{-/-}$ mice in response to chronic arsenic exposure in the presence and absence of HFD. Our data also suggest that females are more protected against arsenic-induced changes in atherosclerotic plaques compared to their male counterparts at this exposure window. The lack of changes in the levels of smooth muscle cells and collagen fibers in females compared to the significant decrease observed in males post arsenic treatment suggest that females have a more stable plaque phenotype, correlating with human data (Man et al., 2020). Men experience fewer stable plaques which results in a higher prevalence of thrombosis and subsequent myocardial infarction and stroke (Man et al., 2020).

3.2 Limitations

3.2.1 The use of animal models

Wildtype mice have a different blood lipid profile than humans, thus they are protected against cardiovascular diseases (Gistera et al., 2022). To study the effects of arsenic-induced atherosclerosis in the context of diets, the use of transgenic mouse models such as the apoE$^{-/-}$ mice was required. There are several mouse models that can be used but

among the most popular animal models for atherosclerosis, apoE$^{-/-}$ mice were the best fitting animal model for our research question due to comparability reasons. Consistent with the literature, we observed larger plaques in females compared to previous data from our laboratory which was consistent in both diet groups (Lemaire et al., 2011). Nevertheless, there are some interspecies differences. In humans, men have bigger plaques whereas in mice, apoE$^{-/-}$ females have bigger plaques, however, there is disagreement among the scientific community that this phenotype disappears as the mice ages (Man et al., 2020; Matsumoto et al., 2016; Zhang et al., 2018). Moreover, mice do not experience plaque rupture, thrombosis and thrombosis-associated myocardial infarction (Man et al., 2020). Investigators have been using surrogate outcomes to assess plaque instability such as necrotic core size, inflammation, and lipid levels, highlighting the importance of assessing plaque constituents rather than just plaque size (Man et al., 2020).

Although there is a chance that these findings might not translate to humans, the use of transgenic animals has been instrumental in understanding disease development and acknowledging these differences allowed us to better interpret our data.

3.2.2 Comparing our data to historical data

There is an ongoing replication crisis in biomedical research (Hunter, 2017). Many studies in health sciences are difficult to reproduce, resulting in controversial results (Hunter, 2017). To use sex as a biological variable, both sexes should be included in the same study at the same time (Man et al., 2020). In this project, we included only females since the males have been studied previously by the Mann lab. Even though the same

protocols, techniques, instruments, and facilities have been used, there might be differences in the exposure due to uncontrollable factors changing over the years such as a water pipe change, introduction of a new substrate in animal cages, etc. We showed the development of arsenic-induced atherosclerosis in female apoE$^{-/-}$ mice, which is still a valuable contribution to science since this was never been shown, however, our data might not be comparable to the historical male data generated by the very same lab.

3.3 Future directions

Previously, our lab sequenced the arsenic-induced atherosclerotic plaques of male apoE$^{-/-}$ mice in the context of HFD using the multiomics approach. Multiome sequencing combines the single cell RNA sequencing (scRNAseq) and transposase-accessible chromatin with sequencing (ATAC-seq) was instrumental to profile the transcriptomic and epigenetic changes in the atherosclerotic plaque induced by arsenic exposure (Mazan-Mamczarz et al., 2022). The data showed that arsenic alters the transcriptional profile of macrophages, creating different subclusters while shifting their interactions with neighboring cells and altering their cellular fates (K. Makhani et al., 2023). A similar approach could be done with female apoE$^{-/-}$ mice. Although, the assessment of arsenic-induced atherosclerotic plaque constituents in females showed minimal changes compared to their male counterparts, following the omics as next step could still be beneficial. Considering the involvement of immune cells in the atherosclerotic plaque development and the chronic inflammatory state of the arteries, this next step could be useful for understanding the role of arsenic in the immune cell heterogeneity, altered cellular functions and interactions. Including the female sex in the omics approach could also yield important insight while interpreting male data.

Moreover, a state-of-the-art staining technique known as the Phenocycler system could be used to physically map these different cell types existing in the arsenic induced atherosclerotic plaques and show cell-cell interactions. The Phenocycler system is a multiplex imaging technique and it has been used stain 100+ markers with the use of oligonucleotide-conjugated antibodies (Jhaveri et al., 2022). Genes that are differentially altered by arsenic exposure can be targeted by this technique to visualize them on a protein level. This technique could also help us spatially understand where the clusters exist on a plaque level while validating cluster-cluster interactions. Considering the complex nature of atherosclerotic plaques, the Phenocycler system is a very efficient and highly multiplex way to perform spatial phenotyping that could shed light on arsenic-specific interactions within the atherosclerotic plaque.

3.4 Concluding remarks

Arsenic is a significant environmental contaminant that affects millions of people over the globe, causing a myriad of adverse health effects but most importantly contributing to the development of leading causes of death, cardiovascular diseases (World Health Organization, 2020c, 2022). Cardiovascular diseases are the number one killer of both women and men, affecting the latter one earlier in life (Man et al., 2020; World Health Organization, 2020c). Sexual dimorphisms emphasize the need for specialized therapies for better treatment and prognosis for both men and women. Instead of a one-size-fits-all approach, therapies targetted based on sex could be more beneficial when treating men and women.

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