The role of arsenic methylation in arsenic-enhanced atherosclerosis

by

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Dedication

I dedicate this thesis to my parents Christina and Luis Carlos

Their unconditional love, support and value in education kept me going. They allowed my wings to grow and helped me when I felt down. They taught me how to be responsible for my choices, resilient in difficult moments and mainly to never give up. For everything that I had accomplished, I had them on my back, cheering and pushing. The journey abroad would have been much more difficult if I had not had their support. I really miss and love them. Meu eterno e sempre muito obrigado.

E por todo amor sempre compartilhado!!!

I wish also to dedicate this thesis to my grandparents

Vó Ada (in memoriam), Vô João, Vó Lourdes (in memoriam), Vô Nico (in memoriam).

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Abstract

Arsenic exposure is a major public health problem worldwide. Chronic exposure through drinking water increases incidence of several diseases. Epidemiologic and experimental studies have shown that arsenic exposure is associated with an increased risk of atherosclerosis. Our lab has been dedicated to understanding the mechanism of arsenic-increased atherosclerosis. The goal of this thesis was to define the role of arsenic biotransformation in the pathogenesis of atherosclerosis. Arsenic is metabolized through a series of oxidative methylation reaction by arsenic (III) methyltransferase (As3MT) to yield methylated intermediates. Using mouse models, we show that methylated arsenicals are pro-atherogenic, and that As3MT is required for arsenic to induce reactive oxygen species and promote atherosclerosis. Importantly, As3MT is expressed and functional in multiple plaque-resident cell types, and transplant studies indicate that As3MT is required in extra-hepatic tissues to promote atherosclerosis. Together, our findings indicate that As3MT acts to promote cardiovascular toxicity of arsenic, and suggest human As3MT single nucleotide polymorphism (SNPs) that correlate with enzyme function could predict those most at risk to develop atherosclerosis from millions exposed to arsenic. However, arsenic exposure scenarios are much more diverse. For example, subjects can be exposed only during development. Pregnancy and development is considered a susceptible window of exposure. Thus, we were interested to assess the potential of arsenicals, at an environmentally-relevant concentration, to enhance atherosclerosis later in life after *in utero* exposure. Using a similar mouse model, we exposed mice from the conception to weaning to arsenicals or tap water and assessed the extent of atherosclerosis after another 13 weeks in the absence of arsenic. Surprisingly, sodium arsenite and methylated arsenical exposure was associated with an increase in plaque formation in the aortic arch and aortic sinus in males and females. In this model, reactive oxygen species were not induced. In addition, we found that in the absence of As3MT, arsenicals failed to enhance plaque formation after *in utero* sodium arsenite exposure. Therefore, our data show that prenatal exposure to inorganic arsenic and methylated-arsenicals have the potential to increase atherosclerosis, which is dependent on As3MT expression. The main focus of the present thesis was to evaluate the impact of arsenic methylation in arsenic-enhanced atherosclerosis. However, arsenic effects are multifactorial. Thus, to better understand the pro-

atherogenic effects of arsenic, we have investigated several mechanisms involved early in the development of atherosclerosis. Monocytes, macrophages, endothelial cells and platelets play an important role in the initiation of the atherosclerotic lesion. We assessed the effects of low-to-moderate arsenic exposure on monocyte adhesion to endothelial cells, platelet activation, and platelet-monocyte interactions. We observed that arsenic induced human monocyte adhesion to endothelial cells *in vitro* and ex vivo. Importantly, both cell types needed to be exposed to arsenic to maximize monocyte adhesion to the endothelium. Arsenic does not activate platelets or enhance platelet/leukocyte interaction. Hence, this adhesion process is specific to monocyte/endothelium interactions. Arsenic increased mononuclear cell adhesion via increased CD29 binding to VCAM-1. Moreover, this result was confirmed *in vivo*, where arsenic-exposed mice exhibited increased VCAM-1 expression on endothelial cells and increased CD29 on circulating monocytes. Interestingly, *in vitro* and *in vivo* antioxidant treatment inhibited arsenic-induced VCAM-1 expression and monocyte/endothelial cell binding. Finally, we have shown that arsenic biotransformation is the key mechanism for arsenic enhanced-atherosclerosis in two different exposure scenarios; supporting the hypothesis that SNPs, predictive of altered As3MT enzyme efficiency in humans, could impact arsenic adverse health effect outcomes. More studies are required to understand the biotransformation process and its consequences on arsenic pathogenesis.

Abrégé

L'exposition à l'arsenic est un problème de santé publique à l'échelle planétaire. L'exposition chronique par l'eau contaminée augmente l'incidence de nombreuses maladies. Des études épidémiologiques et expérimentales ont montré que l'exposition à l'arsenic est associée à une augmentation du risque de développer de l'athérosclérose. Notre laboratoire est étudie les mécanismes d'augmentation de l'athérosclérose induits par l'arsenic. L'objective de cette thèse a été de définir le rôle de la biotransformation de l'arsenic dans la pathogenèse de l'athérosclérose. En effet, l'arsenic est métabolisé par une série de réactions de méthylations oxydatives par une enzyme, l'arsenic (III) méthyltranférase (As3Mt). Ces réactions conduisent à la production de métabolites méthylés. En utilisant des modèles murins, nous avons montré d'une part que les composés arsenicaux méthylés sont pro-athérogènes et, d'autre part, qu'As3Mt est requis afin que l'arsenic induise la production d'espèces réactives de l'oxygène et promeuve l'athérosclérose. Pour la première fois, nous avons établi que l'As3Mt est exprimée et fonctionnelle dans de multiples types cellulaires présents dans l'athérome, et que l'As3Mt est requise dans ces tissus extra-hépatiques pour promouvoir l'athérosclérose. Réunies, nos observations indiquent que l'As3Mt accentue la toxicité cardiovasculaire de l'arsenic, et suggèrent que des SNPs (polymorphisme nucléotidique) humains d'As3Mt corrélant avec sa fonction enzymatique pourraient prédire, sur les millions de personnes exposées à l'arsenic, celles les plus à risque de développer l'athérosclécrose. Les types d'expositions à l'arsenic sont nombreux. L'humain peut par exemple être exposé uniquement pendant son développement, ou uniquement à d'autres moments de sa vie. Des études à hautes doses d'arsenic ont d'ailleurs démontré que l'exposition intra-utérine est une période sensible aux effets délétères de la molécule. Nous avons donc voulu évaluer à une concentration environnementale, la potentialité des composés arsenicaux à augmenter l'athérosclérose plus tard dans la vie, après une exposition *in utero*. En utilisant un modèle animal murin, nous avons exposé des souris à des composés arsenicaux ou à de l'eau du robinet, depuis la conception jusqu'au sevrage. Les animaux ont ensuite été maintenus avec de l'eau du robinet (ne contenant pas d'arsenic) pendant 13 semaines et nous avons évalué la formation et la composition de l'athérosclérose. Nous avons observé que l'arsenite de sodium ainsi que les composés arsenicaux méthylés sont associés à une augmentation

de la formation de plaques dans l'arc aortique et dans le sinus aortique, autant chez le mâle que chez la femelle. Dans ce modèle, la formation des spécimens réactifs d'oxygène n'était pas induite. De plus, nous avons associé l'augmentation de l'athérosclérose à la biotransformation de l'arsenic, car lorsqu'As3Mt était supprimée, les composés arsenicaux n'augmentaient pas la formation de la plaque après exposition des animaux à l'arsenite de sodium. Conséquemment, nos données montrent que l'exposition prénatale à de l'arsenic inorganique et à des composés arsenicaux méthylés ont le potentield'augmenter l'athérosclérose, phénomène dépendant de l'expression d'As3Mt. L'objectif principal de cette thèse était d'évaluer l'impact de la méthylation de l'arsenic dans le mécanisme d'induction d'athérosclérose. Comme les effets de l'arsenic sont multifactoriels, nous avons analysé plusieurs mécanismes impliqués précocement dans le développement de l'athérosclérose afin de mieux comprendre les effets pro-athérogènes de l'arsenic. Les monocytes, les macrophages, les cellules endothéliales ainsi que les plaquettes jouent un rôle important dans l'initiation de l'athérosclérose. Nous avons évalué les effets d'une exposition à l'arsenic, de concentration faible à modérée, dans l'adhésion des monocytes aux cellules endothéliales, dans l'activation des plaquettes ainsi que dans les interactions plaquettes-monocytes. Nous avons observé que l'arsenic induit l'adhésion des monocytes humains aux cellules endothéliales *in vitro* et *ex vivo*. Les deux types cellulaires doivent être exposés à l'arsenic pour maximiser l'adhésion des monocytes à l'endothélium. L'arsenic n'active pas les plaquettes et n'augmente pas les interactions entre plaquettes et leucocytes. Nous avons plutôt observé que le processus d'adhésion en présence d'arsenic est spécifique aux interactions monocytes/endothélium. L'arsenic augmente l'adhésion des cellules mononuclées via l'augmentation de la liaison de CD29 à VCAM-1. Ce résultat a été *confirmé in vivo* dans des souris exposées à l'arsenic, où une augmentation de l'expression de VCAM-1 dans les cellules endothéliales et une augmentation de CD29 dans les monocytes circulants ont été observées. Dans ce modèle, le traitement avec des antioxydants *in vivo* et *ex vivo* a inhibé l'induction par l'arsenic de l'expression de VCAM-1 ainsi que la liaison entre les monocytes et les cellules endothéliales.

Nous avons montré que la biotransformation de l'arsenic est un mécanisme clé dans le développement de l'athérosclérose, lors de deux scénarios d'exposition différents ; ce qui soutient l'hypothèse que les SNPs, prédisant une efficacité altérée de l'enzyme As3Mt chez l'être humain, pourraient avoir des conséquences néfastes sur la santé en présence d'arsenic. D'autres études sont requises pour mieux approfondir et comprendre le processus de biotransformation et ses conséquences sur la pathogénie de l'arsenic.

Acknowledgments

Following a dream and a desire for challenges, I decided to pursue a PhD abroad. The path that brought me here was totally random; so much so that I could not believe it when Dr. Koren Mann emailed me that I had been approved. I cried - Yes, I do have stereotypical Latin emotions.

Doing a PhD is not a single person journey. During the past four years I have had the pleasure to meet and work with so many great people, who if not directly contributing to this work, indirectly contributed to my joy and my motivation to get going. I have learnt a lot, professionally and personally.

First of all, I would like to thank Dr. Koren – my supervisor, mentor and friend. I am not sure how much I believe in luck, but sometimes it is difficult not to. Having the opportunity to be in her lab and work with her was a blessing. I cannot be more thankful for her trust, enthusiasm, guidance and patience with my English (at the beginning and during scientific writing!). She has taught me a lot and also given me the freedom to explore my own ideas and questions. I am sure I will carry her problem solving approach and lessons for the length of my career. The PhD opportunity has changed my life, not just career wise but personally. I owe her my sincere gratitude.

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Finally, I hope the lives that we used in this study were worth it to help society. Unfortunately, science has not yet evolved beyond the use of animal models- we still need them. My sincere sympathy.

List of Abbreviations

ABC	ATP-binding cassette
ABCC	ATP Binding Cassette Subfamily C Member
apoE	apoliprotein E
AQP	aqualipoprotein
arsB	ars opero B
arsC	ars operon C
arsM	As(III) S-adenosylmethionine methyltransferase
arsR	ars operon R
As (III)	arsenite
As(V)	arsenate
As2O3	arsenic trioxide
As3MT	arsenic [1] methyltransferase
AsB	Arsenobetaine
AsC	Arsenocholine
ATCC	American Type Culture Collection
BigET-1	big endothelin-1
BMDM	bone marrow derived macrophages
c-SRC	C-terminal Src kinase
CCL	chemokine (C-C motif) ligand
cGMP	guanosine 3´,5´-cyclic monophosphate
cIMT	carotide intima thickness
CVD	cardiovascular disease
Cys	cysteine
DHE	diihydroethidium
DKO	double knockout
DMA III	dimethylarsinous acid
DMA V	dimethylarsinic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Enviromnental Protection Agency
FBS	Fetus bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GlpF	aquaglyceroporin GlpF
GLUT	Glucose transporte
GSH	glutathione
HDL	high dense colestherol
hERG	ether-a-go-go
HFD	high fat diet
HPLC	High Performance Liquid Chromatography

HUVECs	human vascular endotheial cells
iAS	inorganic arsenic
ICAM-1	intercellular adhesion molecule-1
ICP-MS	Inductively coupled plasma mass spectrometry
IFN-γ	interferon gamma
IL-4	interleukine 4
IP	immuneprecipitation
LDL	low dense lipoprotein
LDLr	low dense lipoprotein receptor
LOX-1	Lectin-like oxidized low-density lipoprotein receptor 1
LPS	Lipopolysaccharides
LXR	liver X receptor
M-CSF	macrophage colony stimulation factor
M0	Macrophage 0
M1	Macrophage 1
M2	Marcrophage 2
MAPK	Mitogen-activated protein kinases
MdR	multidrug resistance
MMA III	methylarsenous acid
MMA V	methylarsonic acid
MMP	metalloproteinase
MOMA	Monocyte/Macrophage marker antibody
mRNA	messenger Riboxyribonucleic acid
microRNA	micro Riboxyribonucleic acid
MRP	Multidrug resistance-associated protein
NAC	N-acetylcyteine
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
ODD	oxidative DNA damage
Oxd.	oxidation
oxLDL	oxidative low dense lipoprotein
PBS	phophate buffered saline
pH	potential of hydrogen
Pit	phosphate inorganic system
ppb	parts per billion
ppm	parts per million
PRMT	protein arginine methyltranferase
Pst	phosphate specific transporter system
qPCR	quantitative polymerase chain reaction
Red.	reduction
RMT	arginine methyltransferase

RNA	Riboxyribonucleic acid
ROS	reactive oxigen species
RXR	retinoic X receptor
SAM	S-adenosyl-L-methionine
SMC	smooth muscle cells
SNPs	single nucleotide polymorphism
TMA	Trimethyl arsenic
TMAO	trimethyl arsenic oxide
TRX	thieoredoxin
US	United States
USA	United States of America
VCAM-1	vascular cell adhesion molecule-1
VEGF	vasccular endothelial growth factor
VLA-4	very late antigen-4
WHO	World Health Organization
WT	wild type

Preface

The following manuscripts are included in this thesis. Detailed contributions are described below.

Chapter 2:

Negro Silva LF; Lemaire M; Lemarié CA; Plourde D; Bolt AM; Chiavatti C; Bohle DS; Vesna Slavkovich V; Graziano JH; Lehoux S; Mann KK. **Arsenic (3) methyltransferase is essential to arsenic-enhanced atherosclerosis.** (Under revision at Environmental Health Perspectives)

Authors contribution: *LFNS – designed experiments, oversaw animal colony and exposure protocol, collected samples, sectioned tissue and stained slides, took pictures, analyzed images, performed tissue culture, western blots and qpCRs, analyzed and interpreted data and wrote the manuscript.* ML,– designed experiments, collected samples, analyzed and discussed data CL – dissected mice and discussed data. DP – prepared arsenic solution for exposure, accompanied the mice colony. AMB – collected samples, analyzed and discussed data. CC- performed some staining and imaged slides. DSB – synthesized MMA III and discussed data. JHG – provided reagents and HPLC-ICP-MS infrastructure, discussed data. VS – performed HPLC ICP-MS and analyzed its data, SL – provided infrastructure, analyzed and discussed data. KKM – designed and oversaw experiments, analyzed and interpreted data, wrote the manuscript and provided funding. All authors reviewed and approved the manuscript before submission.

Chapter 3:

Negro Silva LF; Lemaire M; Lemarié CA; Plourde D; Bolt AM; Chiavatti C; Bohle DS; Goldberg M; Lehoux S; Mann KK. **Prenatal exposure to arsenicals enhances atherosclerosis later in life, which is dependent on As3mt expression.** *(Article in preparation)*

Authors contribution: *LFNS – designed experiments, oversaw animal colony and exposure protocol, collected samples, sectioned tissue and stained slides, took pictures, analyzed images, analyzed and interpreted data and wrote the manuscript.* ML – designed experiments, collected samples and analyzed data. CL – dissected mice and discussed data. DP – prepared arsenic solutions for exposure, accompanied the mice colony. AMB – collected samples, analyzed data discussed data. CC- performed some staining and imaged slides. DSB – synthesized MMA III and discussed data. MG – performed statistical test and discussed data. SL – provide infrastructure, analyzed and discussed data. KKM – designed and oversaw experiments, analyzed and interpreted data, wrote the manuscript and provided funding.

Chapter 4:

Lemaire M*, **Negro Silva LF***, Lemarié CA, Bolt AM, Flores-Molina M, Krohn R, Smits J, Lehoux S and Mann KK. Arsenic exposure increases monocyte adhesion to the vascular endothelium, a pro-atherogenic mechanism. Plos one, v10(9), 2015. (*Equal contribution for the paper)

Authors contribution: ML – designed experiments, oversaw colony and exposure protocol, performed ex-vivo experiments, cultures and adhesion experiments, collected samples, stained and imagined slides, analyzed and interpreted data and wrote the manuscript. *LFNS – designed experiments, collected samples, dissected mice, performed platelets and leucocyte-platelet experiments, took pictures, analyzed images, analyzed, interpreted data and wrote the manuscript.* CL – dissected mice, performed ex-vivo experiments with ML, analyzed, interpreted and discussed data. AMB – collected samples, analyzed and discussed data. MFM – performed some adhesion experiments and stained slides. RMK – provided the lentils diet, performed some experiments and discussed data. JES – provided the lentils diet and discussed data. SL – provide infrastructure, analyzed and discussed data. KKM – designed and oversaw experiments, analyzed and interpreted data, wrote the manuscript and provided funding.

Published co-authored manuscripts not included in this thesis:

Bolt AM, Grant MP, Wu TH, Flores Molina M, Plourde D, Kelly AD, **Negro Silva LF**, Lemaire M, Schlezinger JJ, Mwale F and Mann KK. Tungsten Promotes Sex-Specific Adipogenesis in the Bone by Altering Differentiation of Bone Marrow-Resident Mesenchymal Stromal Cells. Toxicological Sciences, v150(2), 333-46, 2016.

Krohn R, Lemaire M**, Negro Silva LF**, Lemarié CA, Bolt A, Mann KK and Smits J. High-selenium lentil diet protects against arsenic-induced atherosclerosis in a mouse model. The Journal of Nutritional Biochemistry, v27,9-15, 2016.

Bolt AM, Sabourin V, Flores Molina M, **Negro Silva LF**, Plourde D, Lemaire M, Ursini-Siegel J, and Mann KK. Tungsten targets the tumor microenvironment to enhance breast cancer metastasis. Toxicological Sciences, v143(1), 165-177, 2015.

Chapter 1

Introduction

1.1 Why arsenic?

Arsenic is historically considered the king of the poisons and the poison of the kings. However, arsenic harm goes far beyond the ancient politics of conspiracy and murder. Arsenic is a natural, ubiquitous element present in water, soil, rocks, atmosphere and organisms [1].

Arsenic exposure is a major public health problem worldwide. The WHO estimates that more than 100 million people are exposed to concentrations above the recommended limits set at 10 ppb (parts per billion, µg/L) in municipal drinking water in Canada, the USA and several other countries [2]. This problem is not limited to developing countries, as even industrialized nations have highly exposed populations [2-5], which are exposed to concentration over 10 ppb. An exposure threshold of 10 ppb was set primarily based upon cancer outcomes. However, epidemiologic and animal data indicate that even lower concentrations can contribute to increased susceptibility to several other chronic diseases. Recently, the US EPA has started to re-assess the safety standards in water, considering non-cancer outcomes. Specifically, in Canada, high arsenic concentrations have been found in the urine of many Canadians [6]. Therefore, quality data considering exposure to low-to-moderate arsenic concentrations are still necessary to inform public policy and risk assessment. For the purposes of this thesis we considered low-to-moderate concentration a range between 10 to 200 ppb.

The beneficial uses of arsenic and arsenical-related compounds are not within the scope of this thesis. However, of note, arsenic has been used in medicine for over 2000 years. More recently, arsenic trioxide has been used to treat acute promyelocytic leukemia, with amazing results in patients' recovery [7].

1.2 Arsenic geo-distribution.

Arsenic is widely distributed in the earth's crust [4,5]. We will focus on the arsenic present in water, because it is the main source of exposure to humans. Usually, arsenic in the earth's crust is found mainly conjugated with other elements and minerals, such as sulfur, oxygen, iron, copper and lead. The

most important is the conjugation with pyrite, which is one of the most ubiquitous compounds in the environment [8].

Arsenic is considered a metalloid and thus, has both metal and non-metal properties. Arsenic has the highest solubility amongst the heavy metalloids, so it easily forms oxyanion from the rocks and liberates with sulfur in the water [1]. The metalloid concentration in fresh water may vary from <0.1 to 5,000 µg/L [4]. The highest concentrations, usually, are found in groundwater and aquifer, because environmental conditions and water-rock interactions facilitate arsenic mobilization and accumulation [2].

Arsenic distribution is represented in the Figure 1.1 [9]. Because geological composition of rocks is very different, the irregular distribution of arsenic is not surprising. Several environmental conditions enable arsenic dissolution to the water, such as: reducing environments rich in organic matter and microbial activity, oxidizing environments that increase salinity and pH, geothermal waters, sulfide mineralization and mining [4].

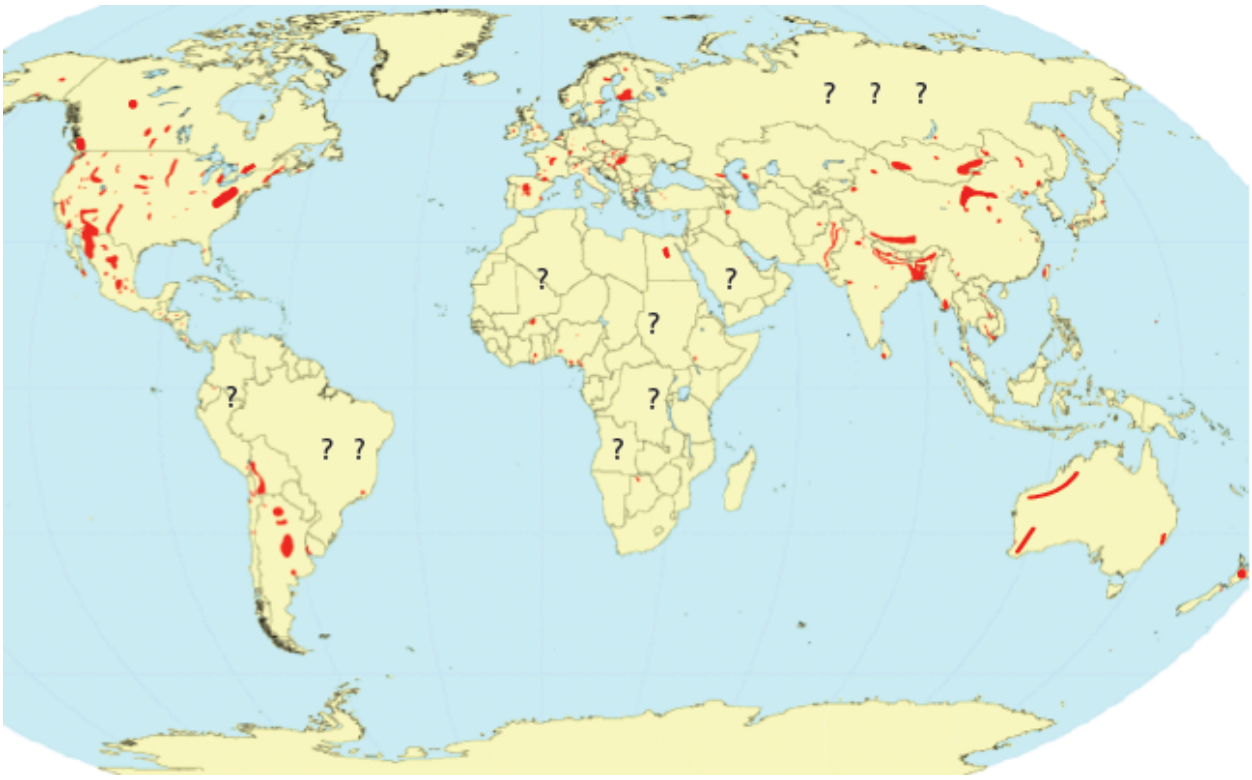

Figure 1.1: Graphic representation of the world map with described hotspots in red for high arsenic concentration in the groundwater, adapted from [9]. *Question mark represents the regions in the respective areas that no arsenic reports were found.*

1.2.1 Human exposure – natural and anthropogenic source.

Arsenic exposure occurs primarily through drinking contaminated water [5, 10]. Major cities have policies and technology to remove arsenic from tap water; however, people who depend on ground water are at risk, mostly because there is no regulation and assessment of private wells. For example, around 30% of the population still depends on ground water as their primary source in Canada [6]. Arsenic has been identified as the major chemical of concern in the US [3]. Interestingly, many states have arsenic-contaminated ground water and several populations exposed [11]. Moreover, many reports demonstrate the relationship between arsenic and increased incidence of several diseases [12-14].

In addition, human activities, including farming with pesticides, mining and some semi-conductor industries, collaborate to increase environmental contamination [15]. Arsenic-based pesticide compounds were banned only in 2006 in the US [16]. Wood preservatives with arsenic are still used. Mining of copper and gold are major sources of anthropogenic arsenic. Particularly, Canada has a massive inactive gold mine contaminated area in Yellowknife, which is under mitigation [17].

There is an increasing awareness of exposure through contaminated food, such as rice and apple juice [18, 19]. Brown rice and rice-based products have been reported to have high levels of arsenic. In Canada, the arsenic intake is driven mainly by food, mostly seafood, although the harm of those compounds is still not clear. Recently, American wine was reported as a source of arsenic, all the collected samples had higher levels than the 10 ppb recommended by US EPA [20].

Most of the industrialized countries have adopted 10 ppb as the maximum contaminant level in water [1, 4]. However, some endemic countries still allow 50 ppb in the drinking water [1]. Usually, highly exposed populations are considered those exposed to above 100 to 200 ppb, which encompasses thousands of millions people around the world [1, 5]. Figure 1.2 exemplifies in a continuum schematic the arsenic concentration in several conditions and area.

Arsenic awareness started in the 1950s with the description of Blackfoot disease in an arsenic rich region in Taiwan; lately, some regions in that country have been characterized as endemic areas [21]. In the 1960s, an event in Chile was reported where people from the village of Antofagasta were exposed to high arsenic concentrations (870 ppb) for about 10 years, after which the exposure abruptly

diminished [22]. Increased incidence of numerous diseases has been described, and interestingly, also later in life when many of the exposures only occurred during childhood or *in utero*.

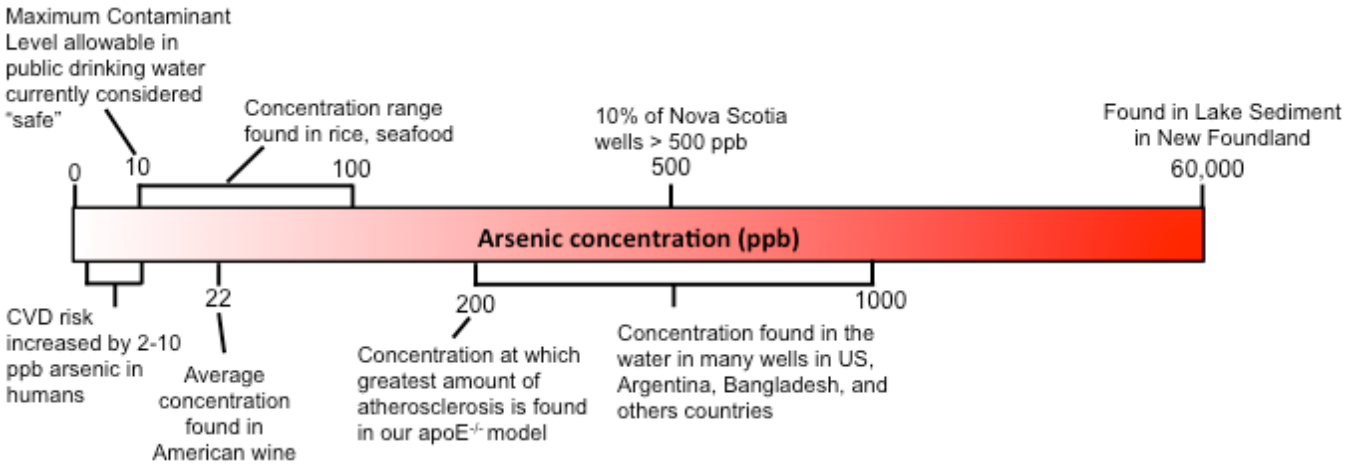

Figure 1.2: Arsenic contamination scheme, which contextualizes arsenic concentration in several conditions, adapted from Dr. Mann design.

Another classic example is in Bangladesh, where a massive exposure occurred. During the 1970s, political forces changed the water source from surface to groundwater, because of the high incidence of water-borne diseases. However, that "clean" water culminated in tens of millions being exposed to high concentrations of arsenic [23].

1.3. Chemistry and metabolism.

1.3.1 Arsenic chemistry.

Arsenic is considered inorganic when associated with other minerals, but organic, if conjugated with hydrogen and carbon. Moreover, arsenic has a wide range of valences, such as -3, 0, +3, +5. Indeed, arsenic species have multiple different behaviors, acting as acids or bases [8]. The most prevalent naturally occurring inorganic arsenic is arsenate [As (V)], which has low mobility because it is adsorbed into the surface of common minerals. Arsenite [As(III)], on the other hand, is more mobile and more prevalent in low oxygen environments. In addition, organic forms (methylated arsenicals) are also found in the environment, as byproducts of microbiota excretion, mammal metabolism or natural breakdown [24]. The formation of methylated forms will be discussed later.

Other complex arsenicals, such as arsenobetaine, arsenosugars and arsenolipids, are also reported. Arsenobetaine is a byproduct of digested arsenosugars in many organisms, mainly zooplankton, which is the base of the food chain of marine animals. Thus, arsenobetaine is found in many seafoods [25]. Arsenosugars and arsenolipids are also found in marine organisms [26, 27]. Although under-studied, those compounds are not considered to be extremely reactive or toxic.

1.3.2 Arsenic metabolism in prokaryotes.

As described, arsenic is a toxicant that naturally occurs in the earth's crust. Consequently, organisms have developed mechanisms to cope with the metalloid. Mechanisms of arsenic resistance have been described from bacteria to mammals. Specifically, bacteria are important for arsenic ecology in the environment [24]. Several bacteria have the *ars operon* of genes, which are responsible for mobilizing, exporting or modifying this metalloid. Figure 1.3 is a schematic presenting the main processes that bacteria have to deal with arsenic [28]. *ArsR*, *ArsB* and *ArsC* are the genes best characterized, which are related with oxidation, regulation and/or export of arsenic.

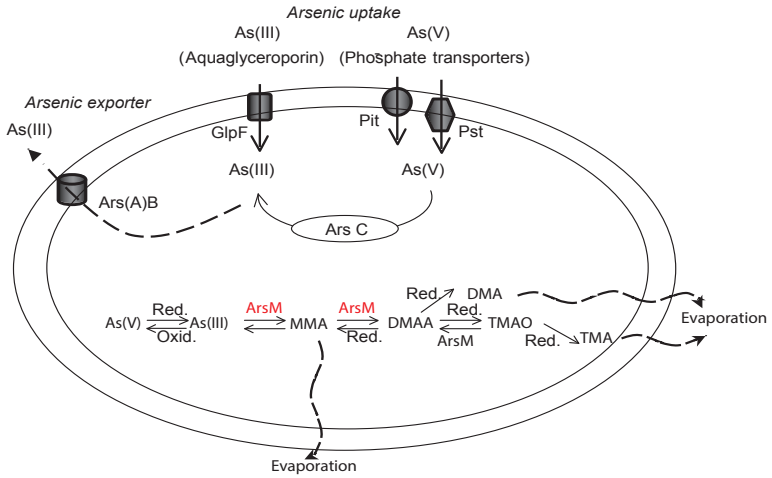

Figure 1.3: Simplified schematic representation of mechanisms that prokaryotes have developed to deal with arsenic, adapted from [29]. Ars(A)B: arsenite efflux pump; ArsC: detoxifying arsenate reductase; ArsM: As(III) S-adenosylmethionine methyltransferase; As (III): arsenite; As (V): arsenate; DMA: dimethylarsine; DMAA: dimethylarsinic acid; GlpF: aquaglyceroporin GlpF; MMA: monomethylarsonic acid; Re.: reduction; Oxid.: oxidation; PiT: phosphate inorganic system; Pst: phosphate specific transporter system; TMA: trimethylarsine; TMAO: trimethylarsine oxide

In addition, ArsM has been described [29], which catalyzes arsenic methylation as indicated in Figure 1.2. The methylation process of arsenic is a series of reduction and oxidation reactions, in which arsenic binds to the cysteines from the active site of the ArsM protein, using S-adenosylmethionine (SAM) as a methyl donor. The biomethylation process produces volatile intermediate compounds, such as monomethylarsonic acid [30], dimethylarsinic acid (DMA) and trimethylarsine oxide (TMAO)[28].

1.3.3 Arsenic uptake in mammalian cells.

Arsenic is imported mainly by promiscuous importers from the Aquaporin family, such as AQP 3, 7 and 9 [31, 32] and also the glucose/fructose transporters GLUT1 and 5 [33]. Some of these importers are described to also import methylated arsenicals [34]. AQP3/9 overexpression significantly augments arsenic toxicity [35, 36]. On the other hand, reduced AQP3 expression causes cells to be more resistant to arsenic's effects [37]. Interestingly, arsenic uptake is impaired by GLUT5 silencing [33]. Moreover, arsenic is also described to be taken up by GLUT1 [38], nevertheless when the pathway of the glucose uptake is blocked or mutated, arsenic still accumulates in the cell [38], suggesting that arsenic is taken up by GLUT1 in a different way than glucose.

1.3.4 Arsenic metabolism in mammals - As3MT structure and function.

Similar to bacteria, mammals have mechanisms to biotransform this metalloid, in order to be more readily exported from the cell. This process involves a series of oxidative-methylation reactions catalyzed mainly by arsenic (III) methyltransferase. The methylation process uses SAM as a methyl donor and can use glutathione (GSH) conjugated with trivalent arsenicals as a substrate [39]. The biotransformation process produces intermediate compounds that include monomethyl-arsenate and -arsenite (MMA V and MMA III, respectively) and dimethyl-arsenate and -arsenite (DMA V and DMA III, respectively). Only in 2002, arsenic (III) methyltransferase (As3MT) was isolated and cloned from the rat liver cytosol [40]. The As3MT enzyme has four cysteines in the activated site, as shown in Figure 1.4, where the arsenic binds to start the methylation reaction. Studies *in vitro* have shown that the activated

cysteines in humans are 61, 32, 156, 206, and mutations in any of these result in a catalytically dead enzyme [41, 42].

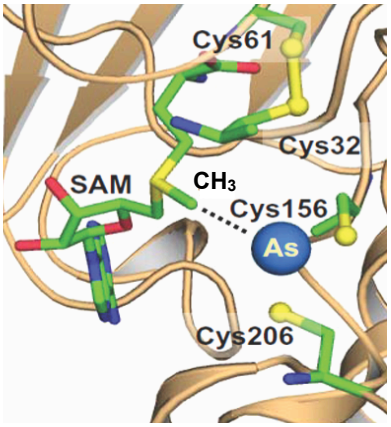

Figure 1.4: Representation of the activity site of hAs3MT enzyme and the SAM with the methyl group to be donated, adapted from [43].

The first pathway to be proposed for the biotransformation of arsenic, shown in Figure 1.5, included a reduction of pentavalent inorganic arsenic to a trivalent inorganic one, followed by oxidative methylation leading to methylated pentavalent arsenic and subsequent rounds of reduction/oxidative methylation producing other intermediates [44]. Figure 1.4 is an adaptation from the first pathway proposed, because at that time none of the methyltransferase family of enzymes had been described.

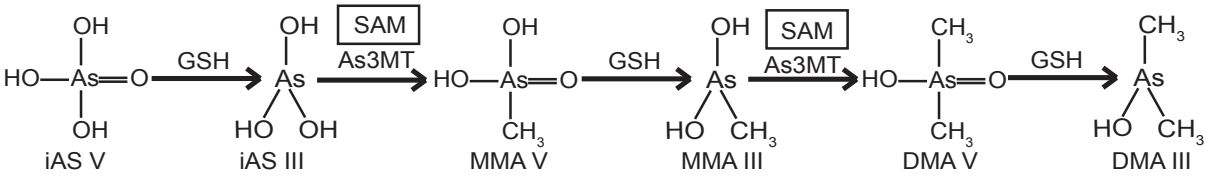

Figure 1.5: First pathway proposed by Challenger 1947, adapted from Hayakawa et al, 2016.

Inorganic arsenic or trivalent forms can conjugate with GSH, and have been demonstrated as substrates for As3MT. As shown in Figure 1.6, the conjugated compounds after methylation are unstable, which hydrolyze and oxidize to the pentavalent forms [45]. The biotransformation catalysis is also supported by other reductants besides GSH. A Trx/TrxR/NADPH coupled system has been described to be required for As3MT activity. This system reduces As(V) to As (III). Moreover, there is evidence that Trx also reduces the methylated arsenic V and cysteine residues in the As3MT enzyme [39].

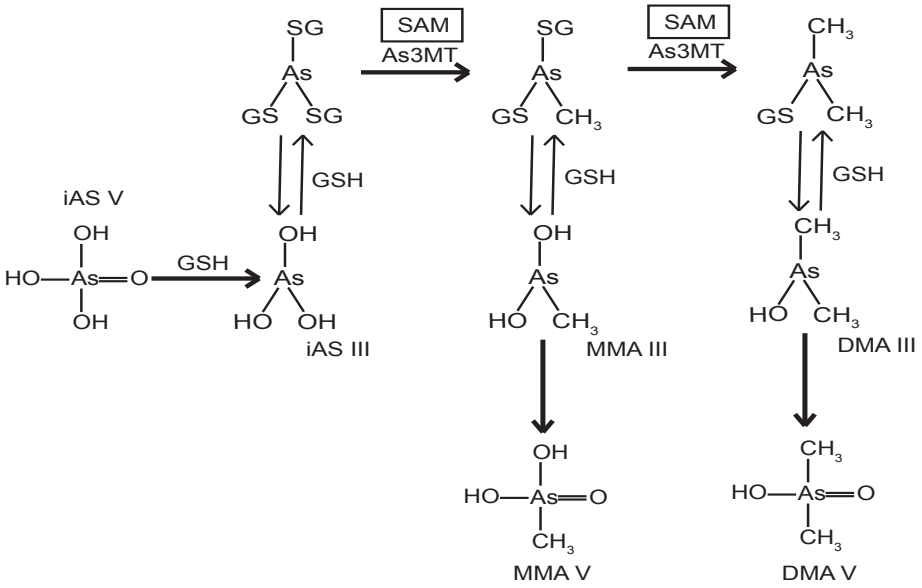

Figure 1.6: Pathway adapted from Hayakawa et al, 2016.

Recently, a mechanism combining both pathways was reported, where As3MT preferentially targets arsenic bound to GSH. Moreover, MMA III remains conjugated to As3MT until the second round of methylation. However, the pentavalent forms were not well defined in this mechanism; they may be obligatory components from the reaction or side products from the oxidation [43].

Although the exact mechanism of arsenic biotransformation is still in debate, there is a consensus about the importance of As3MT in the process. Indeed, As3MT knockout mice show the importance of the methylation process for the excretion of arsenic. The mice have a completely different pattern of retention and distribution of arsenic, with accumulation and excretion of inorganic arsenic and only a slight production of methylated species [46]. Historically, the biotransformation reaction was considered a detoxification process, however, some intermediate species are now recognized as more toxic than inorganic arsenic [47, 48, 49]. The relative contribution of each intermediate arsenical to specific outcomes has not been defined.

1.3.5 As3MT differences between species.

As3MT is well conserved between species. The following table shows the As3MT homology between humans and the main experimental species [50]. Of note, As3MT is not necessarily functional in

all species; for example, chimpanzees have a catalytic inactive enzyme [51]. Interestingly, *Drosophila melanogaster* and *C. elegans* do not have *As3MT* homologues in their genome [39].

Table 1.1: Protein sequence homology between human, macaque, mouse, rat and dog.

Species	Homology
Homo sapiens - Macaca rhesus	97
Homo sapiens - Canis familiaris	83
Homo sapiens - Rattus novergicus	76
Homo sapies - Mus musculus	75

adapted from [51]

Upon ingestion and absorption by intestinal epithelia, arsenic undergoes biotransformation by the process that we have previously described. The biotransformation mainly occurs in the liver [50], however other organs, such as kidney, heart, testes, brain, and adrenal glands also express As3MT. The toxicokinetic contribution of other organs to the arsenic excretion process is not well investigated. Arsenicals travel in the blood stream and freely pass the kidney and are excreted in the urine, or may be conjugated and excreted in the bile/feces.

Interestingly, the extent of arsenic methylation by hepatocytes differs between species. Rats, dogs and macaques are considered to be fast methylators. On the other hand, humans, mice and rabbits are low methylators [50]. The authors claimed that As3MT is highly conserved amongst all those species, and thus, cannot explain the difference in metabolism. Probably, the As3MT protein level determines the variance of the methylation rate observed [50]. The previous statement might not be totally true, because single nucleotide change in humans is known to impact methylation profile [52]. Although rats have been described with a high hepatic As3MT efficiency, arsenic has a longer half-life in this species. This is because the rat's hemoglobin has a chemical structure that facilitates interaction with arsenic, binding mainly DMA III, which retains it longer in the circulation [53].

1.3.6 Arsenic elimination.

Arsenic can be removed from cells as inorganic arsenic, as methylated species, or conjugated with glutathione. ATP-binding cassette (ABC) transporters from the multidrug resistant protein family are described to be critical to arsenic efflux. MRP1 (ABCC1) has been shown to export arsenic and methylated arsenicals conjugated with GSH [54, 55]. Moreover, Mdr1a/1b knockout mice are more sensitive to arsenic toxicity than WT mice [56]. MRP2 has been described as implicated in excretion of similar arsenic compounds in the bile [57]. MRP4 (ABCC4), but not MRP3 and 5, can efflux all arsenicals, including GSH-conjugated species [58], suggesting that this transporter is also a major contributor to arsenic elimination. This is supported by MRP4 expression on the basolateral membrane of the hepatocytes in the liver. Of note, basolateral membrane of the hepatocytes is in contact with the sinusoids, which mediates the uptake and removal of metabolites into the blood stream [59].

Finally, after biotransformation and export from the liver, arsenic is eliminated mainly through the urine. The main form found is DMA, followed by MMA and inorganic arsenic [60]. In fact, these compounds and the ratio between DMA/MMA are assessed to determine metabolism efficiency and exposure in humans. Efficient methylation is defined by a high rate of DMA excretion in the urine. IN many studies, humans that are considered efficient methylators have less severe or lower incidence of disease; on the other hand, partial methylators are related with enhanced disease incidence [61-65]. Moreover, some other compounds, such as trimethyl arsenicals and thioarsenicals, can also be found in the urine.

1.4 Arsenic exposure – a threat to health.

1.4.1 Health issues.

Numerous reports in the literature, based on human and animal models, have shown that arsenic exposure increases many diseases, such as cardiovascular disease [66], impairment of lung [67, 68] and liver function [69], diabetes [70], neurological disorders and several cancers [22] [71, 72]. Moreover, arsenic has been reported to increase death due to chronic diseases [13]. Many studies have evaluated populations exposed to high arsenic concentrations or utilized high concentrations in animal models and cell culture, making it difficult to distinguish the consequences at moderate concentrations. However,

exposure even to low-to-moderate arsenic concentrations considerably increases disease outcomes [66, 73].

Because arsenic and its metabolites are very reactive, a single mechanism of toxicity has not been identified. Instead, arsenic toxicity is very complex and appears to be related to interactions with zinc finger motifs [74], nuclear receptors [75], DNA damage [76-78], autophagy [79, 80] and reactive oxygen species production, by different sources – mitochondria damage [81, 82], NADPH oxidase [83] [84] and endoplasmic reticulum stress [80, 85]. The importance of arsenic biotransformation in the toxicity profile of arsenic has not been extensively investigated.

1.5 Arsenic-induced cardiovascular effects.

Cardiovascular disease (CVD) is the leading cause of death worldwide. Cardiovascular diseases are chronic diseases of the heart and/or blood vessels that can lie undetected for decades. CVDs include coronary heart disease, hypertension, stroke, and peripheral arterial disease. Although better monitoring practices have been developed and some risk factors have been identified, CVD is often only detected late in the disease progression. Risk factors for CVD can be divided into two categories: non-modifiable and modifiable. Non-modifiable include age, gender, and family history, while modifiable include elevated glucose or lipids/cholesterol, obesity, tobacco use, physical inactivity, unhealthy diet, and hypertension (high blood pressure) (http://www.world-heart-federation.org/press/fact-sheets/cardiovascular-disease-risk-factors/). Current data indicate that arsenic exposure could be included as a modifiable risk factor.

1.5.1 Epidemiology.

Arsenic exposure has been linked to CVD for many years, but this link continues to strengthen. Since the 1950s, arsenic exposure was proposed as a cause of Blackfoot Disease, which is characterized by severe peripheral arteriosclerosis [21]. This occlusion of the smaller peripheral vessels results in gangrene, often accompanied by numbness or coldness of extremities and loss of peripheral pulse. The prevalence of Blackfoot Disease decreased dramatically after residents of endemic areas stopped drinking contaminated water [21].

Several studies have linked low arsenic exposure to cardiovascular outcomes. The risk of hospitalization for ischemic stroke is higher in subjects living in areas with ~20 ppb arsenic when compared to those living in areas with less than 4.5 ppb in US [86]. Importantly, people exposed to more than 2 ppb arsenic were more likely to need bypass surgery to circumvent blocked arteries than those exposed to less than 2 ppb [87]. Elevated pulse pressure and mean arterial blood pressure were correlated with drinking water containing an average of 20-30 ppb arsenic in Chinese villagers [88]. This study also showed that the prevalence of increased blood pressure augmented with duration of exposure. In a Spanish study, a small increased risk of coronary heart disease was seen in people exposed to between 1 and 10 ppb arsenic when compared to those exposed to <1 ppb arsenic [73]. More recently, a prospective cohort has shown that Native Americans exposed to low-to-moderate inorganic arsenic have a high rate of cardiovascular disease [66]. Importantly, total urinary arsenic levels in children were associated with increased carotid intima-media thickening; an early marker for atherosclerotic lesions [89], indicating that arsenic may enhance even the early stages of CVD. More recently, a systematic review was performed of numerous epidemiologic studies of arsenic and an increased risk of coronary and peripheral heart disease and stroke. While some studies showed a significant link between arsenic and increased CVD, other studies failed to show a correlation [90]. While the correlation between CVD and arsenic was strong at higher doses, a weaker association was observed at low-to-moderate concentrations, but this may be due to poor study design.

Arsenic may increase CVD by modulating one of the known risk factors. Circulating lipid levels are routinely used as biomarkers for CVD. Increased levels of triglycerides, low density lipoprotein (LDL) or more specifically, oxidized LDL (oxLDL), and cholesterol levels are pro-inflammatory and pro-atherogenic, while high dense lipoprotein (HDL) are anti-inflammatory and anti-atherogenic [91]. However, the data are inconsistent concerning an association between lipid levels and arsenic. Several studies have shown that arsenic exposure did not correlate with triglyceride levels and actually decreased LDL, cholesterol, and HDL levels when compared to non-endemic populations [92, 93]. No association was observed in plasma lipid levels and total urinary arsenic in children [89]. However, increased levels of oxLDL were associated with arsenic-endemic adult populations and increased oxLDL/HDL ratios were

dose-dependent [93]. This suggests that specific pro-inflammatory changes to lipid profiles may correlate with CVD.

In addition to circulating lipid levels, arsenic exposure has been positively associated with several other biomarkers of CVD. Vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) are found on activated endothelial cells and mediate the attachment of leukocytes. Soluble VCAM-1 and ICAM-1 are circulating biomarkers of damaged endothelium. Both VCAM-1 and ICAM-1 levels are increased in arsenic-exposed individuals [93, 94]. Another marker of endothelial activation is Big endothelin-1 (Big ET-1), and arsenic consumption correlates with increased circulating Big ET-1 levels and hypertension [95].

1.5.2 Arsenic and heart function.

Arsenic impairs heart function, mainly resulting in electrocardiographic abnormalities. Prolonged QT interval, which indicates an increased risk of arrhythmia and mortality, has already been recognized as a toxic side-effect of arsenic trioxide treatment against acute promyelocytic leukemia [96]. Assessment of long-term arsenic exposure from drinking water was also associated with QT-interval prolongation in 11% of the analyzed population from the moderate exposure group- 100 to 300 µg/L [96]. Interestingly, there was a sex bias, where women were more affected than men [96, 97]. A US study has shown a positive association between toenail arsenic concentration and interval duration of QT [98]. Molecular mechanisms by which arsenic changes heart electrophysiology are still unclear. One study with arsenic trioxide in a clinical setting reported an effect on the human ether-a-go-go–related gene (hERG) protein, a cardiac potassium channel. Arsenic increases calcium currents by inhibiting hERG-chaperone complexes[99].

1.5.3 Arsenic and hypertension.

In addition to effects on heart electrophysiology, arsenic has been associated to hypertension. Subjects from high arsenic endemic areas are more likely to develop hypertension [100]. More recently, other studies have determined a link between arsenic exposure and hypertension incidence in low to

moderate concentration areas [87, 101]. However, the causal relationship between arsenic exposure and increase in the blood pressure in humans is still unclear [102].

Certainly, experimental studies in mice have indicated this relationship. Female mice chronically exposed to 100 ppb arsenic by drinking water exhibited an increase in systolic and diastolic pressure; those animals also had concentric hypertrophy shown by enhancement in the left ventricular mass [103]. Moreover, *in vitro* studies have described that a high-concentration of arsenic for a short time period inhibits acetylcholine-induced vascular relaxation. Likely, arsenic inhibits NO production in endothelial cells and suppresses smooth muscle cell relaxation by reducing guanosine 3´,5´-cyclic monophosphate (cGMP) [104].

Mechanistic reports showed that arsenic targets endothelial cells and smooth muscle cells (SMCs). For example, SMCs exposed to low doses of arsenic in culture overexpress vascular endothelial growth factor (VEGF), a potent proliferative cytokine [105], which is correlated with remodeling and proliferation of vascular wall.

1.5.4 Mechanisms of arsenic-enhanced atherosclerosis.

The cellular and molecular processes of cardiovascular diseases are complex, involving many cell types and signaling networks. Most studies to define the mechanisms of arsenic-enhanced CVD have focused on atherosclerosis. Atherosclerosis is a chronic inflammatory process of large to medium size arteries. The process is linked with disturbed flow and high-circulating levels of LDL. LDL is oxidized by reactive oxygen species (ROS) to form oxLDL, which accumulates in the sub-endothelial space that activates endothelial cells, recruiting monocytes. The initial inflammatory process also activates platelets that express P-selectin, which facilitates adhesion and chemotaxis of immune cells. Monocytes firmly attach to endothelial cells via adhesion molecules, such as vascular adhesion molecule 1 (VCAM-1) on endothelial cells binding to very late activation antigen-4 ligand (VLA-4) on monocytes, which can then migrate to the intima. Monocytes differentiate to macrophages that phagocytize the oxLDL. Macrophages take up lipoproteins and produce cytokines that recruit T lymphocytes, monocytes and macrophages that maintain the inflammatory process. Once overwhelmed, macrophages cannot efflux all the excess oxLDL, and these lipid-laden macrophages are known as foam cells. Finally, the forming plaque develops a

smooth muscle cell fibrous cap that produces collagen to stabilize the lipid-laden plaques [106-108]. Figure 1.7 is a representation of the atherosclerosis process. The mortality associated with atherosclerosis occurs when plaque breaks off and becomes lodged in a vessel, blocking blood flow to the heart or brain, resulting in myocardial infarction or stroke, respectively [107].

Mouse models of atherosclerosis include the apoplipoprotein E and low density lipoprotein receptor knockout mice, both of which faithfully recapitulate the progression of human disease [109, 110]. High arsenic exposure increases atherosclerosis in both mouse models [111,112]. Arsenic at low-to-moderate concentrations increases the size of atherosclerotic plaque in $ApoE^{-/-}$ mice. Arsenic enhances plaque size in the absence of increasing circulating cholesterol levels [113]. In this model, there is a non-monotonic dose-response relationship between increased atherosclerotic lesion area and arsenic concentration between the low and higher concentration. Arsenic changes the components of the plaque, decreasing smooth muscle cell content and collagen, while increasing lipid within the plaque, such that it has a less stable phenotype that could be more prone to rupture. Interestingly, arsenic does not enhance high-fat diet-induced lesion areas, but it still alters plaque constituents [113], meaning that arsenic is a risk factor for atherosclerosis beyond lipid profile.

Although mechanistic data with low-to-moderate concentrations are limited, arsenic may be acting on several cell types and pathways that result in atherosclerosis. As described above, activation of endothelial cells is an initial event in the formation of atherosclerosis. Arsenic generates reactive oxygen species, particularly hydrogen peroxide and superoxide, in endothelial cells [115], without inducing reactive nitrogen species. At sub-cytotoxic concentrations, arsenic activates oxidant-dependent signaling pathways, including c-SRC, NF-κB, and tyrosine phosphorylation, but not MAPK [116]. Endothelial cells upregulate LOX-1, a receptor that recognizes and imports oxLDL, via ROS- and NF-**κ**B-dependent signaling [117]. All of these signaling pathways are associated with increased adhesion molecule expression on endothelial cells, which contributes to the pathophysiology of atherosclerosis.

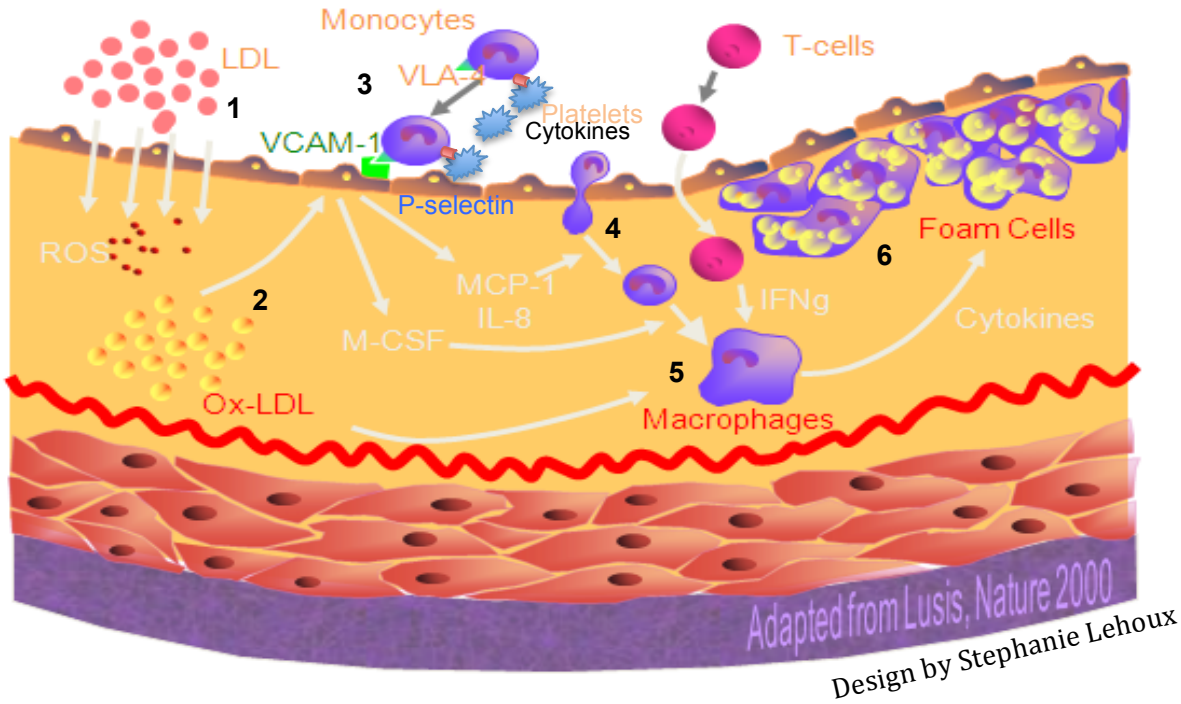

Figure 1.7: Artery cross-sectional representation, showing the classic atherosclerotic process with the main cell players, adapted from the design of Stephanie Lehoux and Lusis [114]. *Note: Numbers indicate the classical timeline process.*

Moreover, vascular remodeling is a critical process in vascular diseases. Indeed, arsenic activates the NADPH oxidase Nox-2 in sinusoidal endothelial cells, inducing capillarization [118], and because this oxidase is found in several vascular tissues, this finding may also be important for the pathogenesis of other systemic vascular diseases induced by arsenic. The same group has shown that arsenic activates vascular remodeling by sphingosine 1- phosphate type 1 receptors. Its stimulation may be the signaling initiation that drives morphological alterations [119].

As discussed, monocytes and macrophages are key players in the development of atherosclerosis. Monocytes and macrophages are both targets for arsenic toxicity. Arsenic inhibits differentiation of human monocytes to macrophages [120], which could decrease the phagocytosis of oxLDL, creating a more pro-inflammatory plaque environment. Macrophages can decrease their lipid load through a process of reverse cholesterol efflux, which is an anti-atherogenic property [109, 121]. Arsenic

exposure leads to accumulation of lipids in macrophages by decreasing expression of genes controlled by the liver X receptor/retinoid X receptor (LXR/RXR) transcription factors, key regulators of the cholesterol efflux [122]. These *in vitro* results have been confirmed *in vivo*, where macrophages express lower LXR/RXR target genes, and correlate with increased plaque [113]. Moreover, apoE$^{-/-}$/LXRα $^{-/-}$ double knockout mice are resistant to arsenic's pro-atherogenic effects [123].

1.5.5 Health outcomes related with prenatal and early life exposure to arsenic.

Until now, we have described the deleterious effects related with arsenic exposure, however most of these reports consider lifelong exposure. Pregnancy has been considered a susceptible window of exposure to toxicants [124]. Pregnant women are recognized as fast "methylators" and arsenic can freely pass through the placenta, thus the fetus is exposed to similar concentrations as the mother [125]. Nevertheless, reports have shown that the fetus is exposed, mainly, to the methylated arsenic form dimethyl arsenical when exposed to inorganic arsenic [125-127].

Recently, cord blood and placenta have been used as a biomarker of low-to-moderate arsenic exposure effects in the fetus. An association between inorganic arsenic exposure, DNA methylation and CD8+ T lymphocyte proportion was observed in the cord blood of infants [128]. Similarly, another report described altered DNA methylation in the white blood cells also from the cord blood [129]. Variation of DNA methylation has been found in placentas from subjects exposed to low concentrations of arsenic [130]. Besides DNA methylation, prenatal arsenic exposure has been reported to alter microRNA expression in the cord blood, which likely regulates innate and adaptive immune response [131]. The challenge of those studies is to determine the causal effects of the observed alteration later in life, such relationships still need to be identified.

Arsenic exposure has several adverse effects on the pregnancy itself. For example, it has been associated with increased risk of spontaneous abortion and other reproductive problems [132, 133]. Moreover, some epidemiological articles have shown arsenic effects in a case-control population in Chile, where subjects were exposed to a higher concentration for over 12 years from the same water source. This event was responsible for an increase in mortality of young adults [22, 134], and increase in mortality due to bladder cancer [12] and myocardial infarction [135]. A cohort in Bangladesh showed that children

exposed to arsenic were at greater risk to die from cancer or CVD [136]. A cross sectional study with children in Mexico associated total urinary arsenic with an augmentation in carotid intima thickness (cIMT) – a marker of subclinical atherosclerosis. The contribution of *in utero* exposure could not be ruled out, though, because most of the mothers lived in the same region during pregnancy [89].

Animal experimentation has been useful in identifying the underlying mechanism of arsenic enhanced atherosclerosis later in life. Indeed, arsenic exposure increases plaque formation in the apoE knockout mice exposed *in utero* [137]. Preprograming in oxidative stress and inflammatory response pathways have been related with enhancement of atherosclerosis later in life, which has been shown by microarray analysis from liver samples of newborns exposed *in utero*. Lipids, stress and inflammatory pathways that may contribute to the plaque formation were also upregulated [138]. Although these pioneer studies demonstrated the late atherosclerotic effects of arsenic exposure *in utero*, the concentrations used were very high (49 ppm), which makes it difficult to extrapolate to human exposure scenarios. Therefore, studies with low-to-moderate concentration are still lacking. Additionally, the impact of biotransformation and methylated arsenicals has never been investigated in this context.

1.5.6 As3MT gene in humans: influence of genetics and arsenic methylation efficiency.

The *As3MT* gene is located on chromosome 10q24, contains 11 exons, and is 32 kilobases in length [52]. Single nucleotide polymorphisms (SNPs) were reported in this gene, which may impact the arsenic methylation pattern. For example, 10% of the analyzed subjects had an amino acid change at Met287Thr in exon 9, which confers an increase of 350% in the allozyme activity [52].

Indeed, several reports have evaluated the consequences of SNPs in the methylation capacity [52, 64, 139-147]. The first report described a population in Mexico where three intronic SNPs were associated with the DMA/MMA ratio in the urine [139]. Another study from Taiwan associated SNP in an intronic region with higher urinary MMA and increased cancer incidence [148]. In addition, a weaker skin lesion association risk has been observed in the fast “methylator” genotype [64].

Moreover, six polymorphisms have been associated with metabolite pattern in two different highly exposed communities. Interestingly, the SNP frequency and methylation pattern were markedly different between these populations. Argentinian Andes women have alleles and haplotypes related with fast

methylation, while Bangladeshi women have alleles associated with inefficient arsenic methylation [149]. The same group suggested that the Argentinians living in the Andes have undergone positive selection to tolerate arsenic [147], because 68% of the subjects have the haplotype considered protective [150]. Most of the literature has related the genetic determinants and methylation efficiency with cancer outcomes. The haplotype considered protective was negatively associated with skin lesions in women [151]. A single polymorphism in As3MT was related with increase in the incidence of premalignant skin lesion [146]. With regard to CVD, lower methylation capacity has been associated with increased risk of fatal and nonfatal CVD, thus genetic difference in subjects may predict who is at greater risk [65]. The same group reported a linear dose-response relationship between urinary MMA% and cIMT, proposing that incomplete methylation directly influences atherosclerosis [152]. Presently, there is no explanation for how arsenic metabolism and methylation affects CVD.

1.6 Rationale for the thesis.

Although well established in literature as a potent toxicant, the arsenic mechanism of action is not totally understood. In regards of cardiovascular disease, the paucity of information is even greater. However, as we have described, arsenic is biotransformed in the body, which may impact the disease pathogenesis due to arsenic exposure. Moreover, several SNPs in populations with altered arsenic excretion efficiency might impact disease susceptibility. The mechanism of how arsenic metabolism impacts cardiovascular disease outcome has never been investigated. Thus, we hypothesize that arsenic methylation is important for arsenic-enhanced atherosclerosis.

1.6.1 Objectives.

The purpose of this thesis was to investigate the role of arsenic biotransformation in arsenic-enhanced atherosclerosis. First, we aimed to determine the impact of As3MT-driven methylation in atherosclerosis. Second, we intended to investigate the atherogenic potential of arsenicals after prenatal exposure and the effect of methylation in the phenotype. Finally, we investigated arsenic's effects on cell-to-cell interaction and activation that are related with atherosclerosis. To this end, animal models, cell culture and bio-molecular techniques were used to execute this study.

1.6.1.1 First Aim.

We have defined the atherosclerotic potential of the methylated arsenicals. Moreover, we investigated how the biotransformation process impacts the outcome. Finally, we explored the mechanism and cell types involved in the phenotype observed. This section of the thesis has been submitted and is presented in the Chapter 2.

1.6.1.2 Second Aim.

As we have described, arsenic exposure during prenatal development may impact disease outcome later in life. Thus, we evaluated the arsenical effects in the atherosclerosis outcome after prenatal exposure. At this project we investigated both sexes using several approaches in animal models. This portion of the thesis is in revision to be submitted for publication and it is presented in the Chapter 3.

1.6.1.4 Third aim.

To investigate the effects of arsenic in the early events of atherosclerosis, we evaluated cellular interactions between endothelial cells, monocytes, platelets and neutrophils, analyzing adhesion molecule expression, conformational changes and cytokine production. This section has been published and it is presented in the Chapter 4.

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Chapter 2

Arsenic (3) methyltransferase is essential to arsenic-enhanced atherosclerosis

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2.1 Abstract.

Arsenic is metabolized through a series of oxidative methylation reactions by arsenic (III) methyltransferase (As3MT) to yield methylated intermediates. While arsenic exposure is known to increase the risk of atherosclerosis, the contribution of arsenic methylation and As3MT remains undefined. Using mouse models, we now show that methylated arsenicals are pro-atherogenic, and that As3MT is required for arsenic to induce reactive oxygen species and promote atherosclerosis. Importantly, As3MT is expressed and functional in multiple plaque-resident cell types, and transplant studies indicate that As3MT is required in extra-hepatic tissues to promote atherosclerosis. Together, our findings indicate that As3MT acts to promote cardiovascular toxicity of arsenic and suggest human As3MT SNPs that correlate with enzyme function could predict those most at risk to develop atherosclerosis from millions exposed to arsenic.

Keyword: arsenic, As3MT, methylation, atherosclerosis, ROS

2.2 Introduction.

Arsenic exposure in humans is recognized as a major public health issue [1, 2], where tens of millions of people worldwide are exposed at concentrations above maximum contaminant levels [3]. Chronic exposure through drinking water increases the mortality rate [4] due to increased incidence of several cancers [5] [6, 7], cardiovascular disease [8], impairment of lung [9] [10] and liver function [11], defective immune responses [12, 13] and diabetes [14]. Of particular concern is the link between arsenic exposure and atherosclerosis. In fact, people exposed to even low concentrations of arsenic are at risk to develop atherosclerosis [8].

Arsenic is biotransformed through a series of oxidation and methylation reactions, primarily catalysed by arsenic (III) methyltransferase (As3MT) [15]. As3MT is conserved from bacteria to mammals [15]. Thus, humans are exposed to methylated intermediates generated by bacteria found in the environment [16]. The methylation reaction uses S-adenosyl-L-methionine (SAM) as the methyl donor and produces intermediate compounds that include both monomethylated (MMA V and MMA III) and dimethylated (DMA V and DMA III) forms of arsenate and arsenite. Several different molecular mechanisms are proposed for the reaction, some involving glutathionylated-arsenic intermediates [17-19]. Regardless of the exact reaction, there is a consensus regarding the importance of the As3MT enzyme in arsenic methylation. As3MT knockout mice have altered retention and distribution of arsenic, with significantly decreased production of methylated intermediates [20]. Historically, this reaction was considered a detoxification process; however, it is now recognized some intermediate species are more toxic than inorganic arsenic [21]. Nevertheless, the relative contribution of each intermediate arsenical to specific outcomes has not been defined.

The capacity to methylate arsenic has been linked to cardiovascular diseases epidemiologically. Lower methylation capacity, indicated by higher urinary MMA% or lower urinary DMA%, was associated with increased risk of fatal and nonfatal cardiovascular disease, including atherosclerosis [22]. The same group reported a linear dose-response relationship between urinary MMA% and carotid intima media thickness (cIMT), a surrogate measure of atherosclerosis, proposing that incomplete methylation influences atherosclerosis [23]. Importantly, human As3MT polymorphisms were linked to differential arsenic methylation efficacy [24]. There was an interaction between several As3MT single-nucleotide

polymorphisms, arsenic content in well-drinking water, and cIMT [25], although this was not statistically significant after adjusting for multiple comparisons. Despite this, these data are suggestive that certain populations may be at greater risk for cardiovascular consequences of arsenic exposure.

We utilized the apoE$^{-/-}$ mouse model to address the role of arsenic biomethylation in arsenic-induced atherosclerosis. Previously, we observed increased atherosclerotic plaque formation in the apoE$^{-/-}$ mouse model after exposure to 200 ppb (parts per billion; μg/L) inorganic sodium arsenite, an environmentally-relevant concentration [26]. Here, we provide data that methylated arsenicals are also pro-atherogenic. Importantly, we show that As3MT activity is required for arsenic-induced atherosclerosis.

2.3 Material and Methods.

2.3.1 Mice.

B6.129P2-*apoE*^{tm1Unc}/J (apoE$^{-/-}$) male mice were obtained from Jackson laboratory (Bar Harbor, ME, USA). As3MT$^{-/-}$ mice (C57BL/6 background) were kindly provided by Dr. David Thomas (US Environmental Protection Agency, Research Triangle Park, NC, USA). ApoE$^{-/-}$As3MT$^{-/-}$ double knockout [27] mice were created in our facility. All experiments were performed with male mice randomly selected from different litters. Purchased mice were acclimatized to housing conditions under a 12-hour light/12-hour dark cycle for at least 2 weeks before experiments. All mice were fed *ad libitum*. The experimental protocol was approved by the McGill Animal Care Committee and animals were handled in accordance with institutional guidelines. McGill Animal Care Committee is certified by the Canadian Council on Animal Care.

2.3.2 Exposure protocol.

Four week old apoE$^{-/-}$ or DKO mice were maintained for 13 weeks on tap water or on tap water containing 200 ppb *m*-sodium arsenite (0.35 mg/L $NaAsO_2$; Sigma-Aldrich, Ontario, Canada), disodium methyl arsonate hexahydrate DSMA (MMA V; 0.78 mg/L; Chem Service, West Chester, PA USA), monomethyl arsenous acid (MMA III; 0.37 mg/L) synthesized as described [28] or cacodylic acid (DMA V; 0.43 mg/L; Sigma-Aldrich, Saint Louis, MO USA). Solutions containing arsenic were refreshed every 2-3 days to minimize oxidation. The mice were fed with AIN-76A purified diet containing 5% fat (by weight) with no cholesterol for all the experiments (Harlan Laboratories Inc, WI, USA), with the exception of one group of DKO mice fed with high fat diet (20% cocoa butter, 0.5% cholesterol, Harlan Laboratories Inc, WI, USA).

2.3.3 Bone marrow transplantations.

Recipient apoE$^{-/-}$ or DKO male mice were lethally irradiated (10 Gy) at 5 weeks of age, and were injected with the donor bone marrow cells 24 hours after the irradiation. Donors were euthanized, and tibias and femurs flushed with PBS. The suspension was passed at least 5 times through an 18-G needle, then through a cell strainer (70 μm), placed into a 50-mL tube, and centrifuged at 1000 rpm for 3 minutes.

The cell pellet was resuspended at 2.5 x 10^7/ml cells, and 200 μl per mouse was injected via the tail vein. After a 4-week recovery period, the animals were exposed to tap water or tap water containing 200 ppb *m*-sodium arsenite. One group of apoE$^{-/-}$ reconstituted with DKO bone marrow was fed a high fat diet.

2.3.4 Plasma analyses.

Blood (0.6 ml) was obtained by cardiac puncture and plasma was isolated using collection tubes (EDTA BD Vacutainer SST, USA). Cholesterol, high (HDL) and low-density (LDL) lipoproteins, triglycerides and liver enzymes - aspartate aminotransferase [14] and alanine aminotransferase [29] were assessed by the Animal Resources Centre (McGill University, Canada).

2.3.5 Atherosclerotic lesion characterization.

The characterization of the atherosclerotic lesions was performed as previously described [26]. Briefly, the fixed aorta was rinsed with ultra pure water, then cut longitudinally and stained *en face* with oil red O (Electronic Microscopy Sciences, PA, USA). Images were acquired using *Infinity Capture* software and camera (Lumenera, Canada). Percentage of lesion area of the aortic arch, as defined as the region from the first intercostal arteries to the ascending arch, was evaluated with the *Image J* software (National Institute of Health, USA; NIH). The atherosclerotic lesions were also evaluated within the aortic sinus from at least 5 animals. Rinsed, fixed and embedded frozen hearts were processed as previously described [26]. Consecutive, 6 μm cryosections were sliced from the aortic base throughout the aortic sinus, where three to five valve sections per animal were stained with oil red O to visualize the plaque areas and were analyzed for their lipid content. Aortic valves were also stained and analyzed for their collagen content (type I and III) using picrosirius red (Polysciences, PA, USA) [26].

2.3.6 *In situ* immunofluorescence.

Smooth muscle cell (SMC) and macrophage content was assessed within the entire plaque area, as previously described [26]. Concisely, aortic sinus sections were rinsed and blocked with 3% bovine albumin serum (Sigma-Aldrich), incubated with primary antibody (1:200 for monoclonal anti-α-smooth muscle cell actin [clone 1A4], 1:50 for moma-2 [Abcam, MA, USA]), rinsed and incubated with

fluorescently-labelled secondary antibodies (1:500; Invitrogen, ON, Canada). The presence of the immunofluorescent marker from at least 3 sections per animal was quantified using *Image J* software (NIH) and expressed as percentage of the total lesion area.

2.3.7 *In situ* dihydroethidium staining.

Frozen aortic sinus sections were stained with 2 μM dihydroethidium (ThermoFisher Scientific) in PBS and analyzed immediately using a fluorescent microscope. The presence of the fluorescent marker from at least 3 sections per animal was quantified using *Image J* software and expressed as percentage of the total vessel area.

2.3.8 Primary bone marrow-derived macrophage (BMDM) differentiation and polarization.

Bone marrow from apoE$^{-/-}$ or DKO mice were isolated from the femur and tibia of 10-12 week old mice. Bones were flushed with RPMI 1640, and a single-cell suspension was created by passing it 5 times through an 18G needle. The cell suspension was filtered and centrifuged at 1000 rpm for 3 min. Pelleted cells were suspended in RPMI-1640 (Wisent, Inc) + 10% FBS (Wisent, Inc) plus penicillin/streptomycin (Wisent, Inc) and plated to perform a monocyte-enriching adherence step for 1 hour. Next, non-adherent cells were removed and fresh media was added with 50 ng/mL of M-CSF (Peprotech Inc, USA) and cultured for 5 days. Media was refreshed every 2-3 days. After 5 days, cells were kept in M-CSF for M0 or polarized towards M1 (50 ng/mL IFN- γ, Peprotech Inc, USA) or M2 (10 ng/mL IL-4, Peprotech Inc, USA) for an additional 48 hours.

2.3.9 Staining for cellular lipid accumulation.

Unpolarized (M0) BMDM from apoE$^{-/-}$ or DKO mice were isolated as described above and cultured on cover slip for 5 days in M-CSF with or without of $NaAsO_2$. Then, cells were exposed to 2.5μM of 7-ketocholesterol for a further 24 hours. Next, cells were stained with oil red O for 40 minutes, counter-stained with Hematoxylin for 2 min, and rinsed with PBS before mounting the slides. Images were acquired using *Infinity Capture* software and camera. At least 200 macrophages in 2 different fields were counted and the results were expressed as percentage of oil red O positive cells per total cell number.

2.3.10 Endothelial cells isolation and culture.

This protocol was adapted from [30]. Endothelial cells were isolated from lungs of 8-10 week-old apoE$^{-/-}$ or DKO mice. The lungs were cut into small pieces and incubated at 37°C for 60 minutes in 0.1% collagenase A (Roche Diagnostics) in RPMI plus penicillin/streptomycin. The suspension was transferred into a 50-mL tube and passed 15 times through an 18-G needle, then through a cell strainer (70 µm) and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and cells plated in 75-cm^2 tissue culture flasks coated with 0.1% gelatin (Millipore) and grown for 1 week in 50% DMEM/F12 (Wisent, Inc) supplemented with 10% FBS and penicillin/streptomycin and 50% EGM-2 single quote media (Lonza). Endothelial cells were selected by 2 consecutive immuno-isolation steps using magnetic beads conjugated with anti–ICAM-2 antibody (BD Biosciences PharMingen) and plated into 75-cm^2 tissue culture-gelatine coated flasks. Endothelial cells were used for experiments at passage 4. Cultures were serum- starved for 24 hours prior to experiments.

2.3.11 Smooth muscle cell isolation and culture.

SMC were isolated from the thoracic aorta of 8-10 week-old apoE$^{-/-}$ and DKO male mice [31]. After removal of the adventitia, the tunica media was digested with 3 mg of collagenase (Worthington Biochemical Corporation, NJ, USA) and 3 mg of elastase (Sigma-Aldrich) for 0.5 h at 37 °C. The suspension was passed at least 10 times through an 18-G needle, then through a cell strainer (70 µm) and placed into a 50-mL tube and centrifuged at 2000 rpm for 2 minutes. The supernatant was removed and cells plated into 25-cm^2 tissue culture flasks in DMEM (Wisent, Inc) supplemented with 10 % fetal bovine serum and penicillin/streptomycin for 2 weeks. Experiments were performed at passage 3 or 4. Cultures were serum-starved for 24 hours prior to experiments.

2.3.12 *In vitro* dihydroethidium staining.

Primary cells were isolated and culture as described above. Cells were stained as described elsewhere [32]. Briefly, BMDM, SMC or endothelial cells were stained with 5 µM dihydroethidium in media for 10 min, after which 200 ppb $NaAsO_2$ was added for a further 30 min. Rotenone (Sigma Aldrich) at 10µM and Menadione (Sigma Aldrich) at 200µM were used as positive controls. The images were

analyzed immediately using a fluorescence microscope. The relative fluorescence was used to determine changes in dihydroethidium oxidation normalized to the number of cells in the field.

2.3.13 Gene expression.

Total RNA was isolated from BMDM using RNeasy (Qiagen, Hilden, Germany). cDNA was prepared from total RNA using the iScript cDNA synthesis kit (Bio-rad, Mississauga, ON, Canada). Gene expression was analyzed by qPCR using the Applied Biosystems 7500 Fast RT-PCR system, using Fast SYBR Green Master Mix (Life Technologies). Experiments were performed using qPCR primers designed and purchased from Integrated DNA Technologies (Coralville, IA, USA): m36B4 (5'TCATCCAGCAGGTGTTTGACA3' and 5'GGCACCGAGGCAACAGTT3'), As3MT (5'GAAAACTGCCGAATTTTGGA3' and 5'GCCGTGGAGAAAAGTCACAT3'). Experiments were performed using three technical replicates. Data were normalized to the housekeeping gene m36B4. Fold change in gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using naïve gene expression as the reference sample.

2.3.14 Protein quantification and Immunoblotting.

Cells were lysed using RIPA buffer (50mM Tris-HCl, pH 8.0, with 150mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) at 4°C. After sonication, cell lysates were centrifuged at 13,000 rpm for 10 minutes. The supernatants were collected and protein concentrations were quantified with the Bio-Rad protein assay (Bio-Rad, Mississauga, ON). Samples were boiled at 95°C and equal amounts of protein (50 µg) were loaded on 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, CA). Membranes were blocked for 30 minutes with 5% milk/TBS plus 10% Tween 20, and probed with the primary antibody overnight at 4°C. Next, blots were incubated with anti-rabbit antibodies (BD PharMingen) for one hour at room temperature, the signals of targeted protein were developed with chemiluminescence substrate (Amersham, GE Healthcare, Buckinghamshire, UK). GAPDH or Lamin A was used as endogenous control to confirm equal protein loading.

2.3.15 Antibodies.

As3MT polyclonal antibody was raised in rabbit using the following synthetic peptide (EpiCypher, Research Triangle Park NC, US): [C]HGRIEKLAEAGIQSESYDIV. The amino acid in brackets was added to improve solubility of the peptide and for coupling. Further, the peptide was conjugated to keyhole limpet hemocyanin (Sigma-Aldrich) and sent to Pocono Rabbit Farm and Laboratory Inc (Philadelphia US) for antibody production. Rabbit serum was purified by affinity with antigen column using SulfoLink immobilization kit (Thermo Scientific). Commercially antibodies were used: anti-rabbit GAPDH (Life Science), anti-rabbit Lamin A (Santa Cruz).

2.3.16 Arsenic speciation in BMDM.

Media from BMDM were collected after 48 hours of exposure to 50ppb $NaAsO_2$. Media were diluted in 0.1 volume 150 mM aqueous mercury chloride to displace trivalent As from protein thiols. After keeping these samples on ice for one minute, they were deproteinized by mixing with one volume of 0.66 M ice cold $HClO_4$ and centrifuged for 10 minutes at 4000 rpm. The supernatant was sent to Columbia University (New York) for analysis. Briefly, the supernatant was mixed with mobile phase buffer and injected onto the HPLC column, connected to the ICP-MS-DRC. Calibration standards of As metabolites mixture were treated the same way to achieve the same pH and composition as deproteinized samples.

ICP-MS-DRC (Perkin-Elmer) was coupled to HPLC and used as a detector for six arsenic metabolites chromatographically separated on Anion Exchange, using a Hamilton PRP-X100 column with 10mM Ammonium nitrate/Ammonium phosphate, pH 9.1, as mobile phase. Excellent separation power by HPLC, coupled with very low detection limits of ICP-MS-DRC allowed to detect AsC, AsB, MMA, DMA, As3+ and As5+, without on-line digestion of organic forms, with great precision with down to total As concentrations of 0.1 µg/L.

2.3.17 Statistical considerations.

For statistical analysis, the one-way ANOVA was performed and the p value was evaluated with a Dunnett's or Bonferroni Post-hoc test using the GraphPad Instat software (San Diego, CA, USA). A p value <0.05 indicated statistical significance. The data correspond to the mean values ± S.D.

2.4 Results.

2.4.1 Methylated arsenical exposure enhances atherosclerosis and alters plaque components in the apoE$^{-/-}$ mouse model.

Moderate (200 ppb) concentrations of sodium arsenite are pro-atherogenic, in apoE$^{-/-}$ mice, such that they increase plaque formation and alter the plaque components after 13 weeks of exposure through the drinking water [26]. However, the potential effects of organic arsenicals in our system were unknown. Thus, we compared the methylated arsenicals, MMA V, MMA III and DMA V, with inorganic arsenic ($NaAsO_2$) by exposing apoE$^{-/-}$ mice to 200 ppb of individual arsenicals for 13 weeks in the drinking water. The plaque formation was accessed *en face* in the aortic arch after staining with oil red O. Surprisingly, when compared with the control group, exposure to all the methylated arsenicals enhanced the size of the atherosclerotic lesion (Figure 2.1A). We further analyzed the plaque size and components in cross sections of the aortic sinus. Here, although we observed increased plaque size in all groups, only $NaAsO_2$ was statistically significant (Figure 2.1B; p= 0.07 for MMA V vs control). We previously observed that $NaAsO_2$ exposure increased lipid content within the plaques, without a concomitant increase in macrophage number, suggesting that each macrophage accumulates more lipids per cell [26]. Similar analyses for lipid content by oil red O staining within plaques showed that methylated arsenicals increased lipids to the same extent as $NaAsO_2$ (Figure 2.1C), despite no change in macrophages per plaque area as assessed by immunofluorescent staining (Figure 2.1D). We also evaluated collagen and smooth muscle cells (SMC), an increase in which promotes plaque stability [33, 34]. Collagen content was reduced in groups exposed to $NaAsO_2$- and monomethylated arsenicals (MMA V and MMA III), but not in the DMA V-exposed groups (Figure 2.1E). Interestingly, SMC content was decreased in all arsenic-exposed group, reaching statistical significance only in $NaAsO_2$-exposed mice (Figure 2.1F).

Arsenobetaine is also an organic arsenical and is the main arsenic-containing compound found in many fish [35] [36]. However, it is not biotransformed by As3MT in laboratory animals [37]. We tested whether arsenobetaine also acted as a pro-atherogen. Interestingly, arsenobetaine exposure did not increase plaque formation in the arch or sinus (Figure 2.1A and 2.1B). Together, these observations indicate that methylated arsenicals are pro-atherogenic and can cause changes in plaque components in

a manner similar to that of $NaAsO_2$. Further, the lack of pro-atherogenic activity by arsenobetaine suggests that biotransformation by As3MT is important.

2.4.2 As3MT deletion prevents arsenic-induced atherosclerosis.

To study the relative contribution of As3MT in arsenic-induced atherosclerosis, we developed a double knockout mouse model by crossing $As3MT^{-/-}$ mice with $apoE^{-/-}$ mice. The $As3MT^{-/-}/apoE^{-/-}$ double knockout [27] mice were viable, and born with 1:1 a sex ratio with no difference in litter size as compared to $apoE^{-/-}$ mice (Supplementary Figure 2.1A). We confirmed that the lack of As3MT expression in liver extracts from DKO mice, with and without $NaAsO_2$ exposure (Supplementary Figure 2.1B), as well as the lack of arsenic methylation by measuring methylated intermediates in the urine (Supplementary Figure 2.1C). No differences were observed in the body weight (Supplementary Figure 2.1D and 2.1E), circulating lipid profiles, liver enzymes, or complete blood count (Supplementary Tables 2.1 and 2.2) as compared to $apoE^{-/-}$ mice.

We then compared $apoE^{-/-}$ and DKO mice exposed to tap water or 200 ppb $NaAsO_2$, MMA III, or DMA V for 13 weeks, and measured the extent of atherosclerotic plaque. In contrast to $apoE^{-/-}$ mice, the DKO mice exposed to $NaAsO_2$ exhibited no increased plaque formation in either the aortic arch (Figure 2.2A) or the aortic sinus (Figure 2.2B) as compared to tap water exposed mice. Moreover, exposure to methylated intermediate arsenicals did not rescue the phenotype (Fig 2A and 2B), which suggests that the arsenic methylation process is driving atherosclerosis. Importantly, DKO mice were specifically protected from arsenic-enhanced atherosclerosis, because high-fat diet (HFD) enhanced plaque formation in these animals (Figure 2.2A and 2.2B). Of note, in the aortic sinus, control DKO mice had slightly more plaque area than control $apoE^{-/-}$ mice (Figure 2.2B), although this finding was not observed in the aortic arch.

Next, we evaluated whether DKO mice were also protected from arsenic-induced changes in plaque composition. In $apoE^{-/-}$ mice, both inorganic and methylated arsenicals increased lipid accumulation per macrophage, with concomitant decreases in smooth muscle cells and collagen (Figure 2.1C-F). In contrast, the DKO mice exposed to arsenicals exhibited no decrease in the number of SMC (Figure 2.2C), and only $NaAsO_2$-exposed mice exhibited reduced collagen content (Figure 2.2D).

Surprisingly, plaques from control, un-exposed DKO mice had less smooth muscle cell and collagen than control apoE$^{-/-}$ (Figure 2.2C and 2.2D). This correlated with the small increase in lesion area observed in the sinus of DKO mice.

Arsenical exposure did not increase lipid content (Figure 2.2E) or macrophage infiltration (Figure 2.2F) in the plaques of DKO mice. In addition, the lipid and macrophage content in plaques of un-exposed DKO mice was not significantly different from control apoE$^{-/-}$ mice. These data suggest that arsenic methylation is required for arsenic-induced lipid accumulation in macrophages. Considering our previous data implicating macrophage lipid homeostasis as an important target for arsenic [26, 38], we further investigated the role of As3MT in arsenic-induced macrophage lipid accumulation *in vitro*. We cultured bone marrow-derived macrophages (BMDM) from apoE$^{-/-}$ and DKO mice. BMDM were exposed to 0.1 and 0.6 μM $NaAsO_2$ (corresponding to 10 and 50 ppb $NaAsO_2$) for 5 days during differentiation with M-CSF, then challenged with 7-ketocholesterol for another 24 hours. Consistent with what we observed *in vivo*, $NaAsO_2$ increased the number of apoE$^{-/-}$ macrophages that stained positive for oil red O, indicating an increased lipid accumulation in response to arsenic (Figure 2.2G). However, the DKO BMDM were protected against arsenic-increased lipid loading (Figure 2.2G). These findings suggest that arsenic methylation by As3MT is required for arsenic-induced atherosclerosis. Moreover, As3MT expressed in macrophages is required for arsenic-mediated effects on lipid handling.

2.4.3 Arsenic methylation is necessary for reactive oxygen species production.

Increased reactive oxygen species (ROS) is a well-reported consequence of arsenic exposure [32, 39]. Furthermore, we have shown that inorganic arsenic induces ROS-mediated monocyte/endothelial cell interactions, an early event in atherosclerosis [40]. Supplementation with the anti-oxidant selenium decreases arsenic-enhanced atherosclerosis [41]. Therefore, we postulated that ROS may be differentially produced between the apoE$^{-/-}$ and DKO mice following arsenical exposure. Thus, we performed dihydroethidium [19] staining to detect ROS in aortic sinus cross-sections from apoE$^{-/-}$ and DKO mice exposed to 200 ppb arsenicals or tap water for 13 weeks. All apoE$^{-/-}$ groups exposed to arsenicals had significantly increased intensity of DHE staining per vessel area when compared to controls (Fig 3A). Surprisingly, neither inorganic nor methylated arsenicals induced ROS in the absence

of As3MT expression (Fig 3A). We then investigated whether specific cell types within the plaque were deficient in ROS production in the DKO mice *in vitro*. Thus, primary macrophages, SMCs, and endothelial cells were exposed to 200 ppb $NaAsO_2$ for 30 minutes, stained with DHE, and then analyzed using a fluorescence microscope. Interestingly, macrophages (Figure 2.3B), endothelial cells (Figure 2.3C) and SMCs (Figure 2.3D) produced ROS in response to $NaAsO_2$ when derived from $apoE^{-/-}$ mice, but not when derived from DKO mice. Nevertheless, DKO-derived macrophages were not resistant to ROS induced by rotenone or menadione (Supplementary Figure 2.2). These data indicate that As3MT expression and/or the methylation process is required for arsenic-induced ROS in multiple cell types.

2.4.4 Macrophages, smooth muscle cells and endothelial cells express As3MT.

Clearly, As3MT deletion inhibits arsenic-induced ROS and atherosclerosis following exposure to either $NaAsO_2$ or methylated arsenicals. As3MT is highly expressed in the liver, but very little is known about which cell types in the atherosclerotic plaque express As3MT and whether it is functional. Deletion of As3MT reduces ROS production in the sinus, thus, we postulated that plaque-resident cells could express As3MT and produce methylated intermediates *in situ*.

Plaque-resident macrophages are heterogeneous, where the inflammatory M1 and alternative wound-healing M2 polarization states are the best described in literature [42]. Thus, we evaluated the expression of As3MT in BMDM differentiated and polarized *in vitro*. Macrophages were cultured with classical stimuli for polarization: M0 (M-CSF), M1 (IFN- γ) and M2 (IL-4). After polarization, cells were exposed to vehicle or 50ppb $NaAsO_2$ for 24 hours. As3MT mRNA was detected in all three polarized BMDMs, although at a much lower level than the expression in murine liver (Figure 2.4A). Next, protein expression was determined by western blotting using a rabbit polyclonal antibody that we produced. As3MT protein could be detected in all macrophage subtypes (Figure 2.4B). In addition, As3MT protein was expressed in smooth muscle cells and endothelial cells (Figure 2.4C). Of note, exposure to $NaAsO_2$ did not alter As3MT protein expression levels. Finally, using HPLC/ICP-MS, we determined the capacity of these cell types to methylate arsenic. Methylated forms of arsenic were detected in the media from BMDM (Figure 2.4D) after arsenic exposure. Together, these data demonstrate that macrophages,

smooth muscle and endothelial cells express functional As3MT and consequently, local arsenic methylation might be important for the pathogenesis of arsenic-induced atherosclerosis.

2.4.5 Arsenic-induced atherosclerosis is driven by As3MT-dependent mechanism, in the host and bone marrow compartment.

Based on our data showing that As3MT is expressed in macrophages, endothelial cells and smooth muscle cells, we investigated the relative contribution of bone marrow-derived versus non-bone marrow-derived As3MT on arsenic-induced atherosclerosis. We carried out bone marrow transplantations where apoE$^{-/-}$ or DKO male mice were irradiated and reconstituted with either apoE$^{-/-}$ or DKO bone marrow. After 4 weeks of reconstitution, mice were kept on tap water or transferred to 200 ppb $NaAsO_2$ for another 13 weeks. ApoE$^{-/-}$ transplanted with apoE$^{-/-}$ bone marrow and exposed to 200 ppb $NaAsO_2$ showed increased plaque formation when compared to tap water controls (Figure 2.5A), which recapitulated the results that we have published [26]. Furthermore, DKO transplanted with DKO bone marrow were protected against arsenic-induced atherosclerosis. Interestingly, neither apoE$^{-/-}$ transplanted with DKO bone marrow nor the DKO reconstituted with apoE$^{-/-}$ bone marrow had increased lesion size after $NaAsO_2$ exposure compared to their respective controls (Figure 2.5A). We did observe that apoE$^{-/-}$ mice transplanted with DKO bone marrow had a significantly increased basal level of plaque compared to apoE$^{-/-}$ transplanted with apoE$^{-/-}$ (Figure 2.5A). DKO mice reconstituted with DKO bone marrow also had slightly elevated levels of plaque size consistent with what we observed in figure 2.2. Lipid (Figure 2.5B) and smooth muscle cell (Figure 2.5D) content were reduced only in apoE$^{-/-}$ transplanted with apoE$^{-/-}$. Collectively, the data reveal that both host- and bone marrow-derived cells participate in arsenic methylation and are mandatory for arsenic-induced atherosclerosis.

2.5 Discussion.

Epidemiologic studies have linked arsenic exposure to increased cardiovascular disease. However, the contribution of arsenic biomethylation is unclear. We utilized our mouse model of arsenic-enhanced atherosclerosis to investigate the impact of the methylation process. We show that methylated arsenicals are pro-atherogenic, and like inorganic arsenic, alter plaque components towards a more unstable, rupture-prone phenotype. We also demonstrate that only arsenicals that undergo biotransformation are pro-atherogenic, supported by data showing that arsenobetaine does not increase plaque formation. Furthermore, we show that apoE$^{-/-}$ mice that also lack the enzyme that catalyzes arsenic methylation, As3MT, are protected from arsenic-, but not high fat diet-, induced atherosclerosis. Our data also support the conclusion that arsenic biotransformation is not necessarily a detoxification process, but rather is required for its pro-atherogenic effects.

Humans are exposed to methylated arsenicals [16], but their impact on public health has been underestimated and under-evaluated. Methylated arsenicals are found in the air [43], soil [44] and water [45] [46] [47]. The relative toxicities of methylated arsenicals have been investigated *in vitro* and *in vivo*. MMA III is more cytotoxic than inorganic arsenic or DMA in cell culture systems [21]. Similar results were observed in an endothelial cell line, where inorganic arsenic and MMA III$(GS)_2$ were more cytotoxic than MMA V and DMA V [48]. MMA III and inorganic arsenic inhibit differentiation of embryonic stem cells to the same degree, while DMA III had reduced effects [49]. *In vivo*, methylated arsenicals can cause hyperplasia in urothelial cells [50]. Specifically, MMA III induces proliferation and tumorogenesis in an urothelial human cell line [51]. DMA V-exposed rats pre-treated with carcinogens developed a broad range of tumors in different organs [52]. To our knowledge, this is the first study to show non-cancer related effects of low-moderate concentration of methylated arsenicals *in vivo*.

Our data show that not only are methylated arsenicals pro-atherogenic, but that As3MT is required for the pro-atherogenic effects of arsenic in the apoE$^{-/-}$ model. This result suggests that alterations in As3MT function could be correlated with the risk of atherosclerosis. Several genetic polymorphisms in the As3MT gene have been described in humans, some of which alter the enzymatic efficiency [24, 53-55] [56, 57]. Indeed, arsenic methylation capacity, as measured by the presence of methylated arsenicals in the urine, is inversely associated with risk of developing disease, including

cardiovascular disease [22, 58]. As3MT SNPs are associated with atherosclerotic markers in humans, although these data were not significant after correcting for multiple comparisons [25]. Our results support the hypothesis that methylation efficiency, not particular intermediate forms, correlates with the risk of deleterious arsenic effects, and should be re-evaluated in multiple cohorts.

Macrophages are the main cell type that uptake and accumulate lipids at the atherosclerotic site, and are required for generation of the atherosclerotic plaque [59, 60]. Our previous data implicated macrophages as key cellular targets in arsenic-enhanced atherosclerosis, through increased lipid accumulation due to inhibition of efflux pathways controlled by the liver X receptor [38, 61]. We now extend these data by showing that arsenic methylation is essential for arsenic-induced lipid accumulation in macrophages. Furthermore, our transplant studies support the concept of extra-hepatic metabolism of arsenic, because As3MT expression was required in both the hematopoietic and non-hematopoietic tissue to support the full pro-atherogenic effects of arsenic. Indeed, we found that As3MT is expressed and functional in several of the key cell types in the atherosclerotic plaque: macrophages, endothelial cells, and smooth muscle cells. These cell types are known to be targets of arsenic toxicity [32, 62, 63] [40, 64-66] [67] [68]. This leads to the intriguing possibility that arsenic methylation can occur at the site of atherosclerotic lesion, rather than only occurring in the liver and methylated arsenicals traveling to the lesion. Generation of a floxed-As3MT mouse is required to delineate which cells contribute to arsenic-methylation in the plaque leading to atherosclerosis.

Arsenic is well known to stimulate ROS production [32] [69-71]. The extent of arsenic-induced atherosclerosis correlates with ROS within the plaques [41]. Here, we provide evidence that arsenic methylation is required for arsenic-induced ROS production *in vitro* and *in vivo*. Arsenic metabolism is required for arsenic-induced oxidative DNA damage (ODD) in murine and human cell models of carcinogenicity [72]. However, the same group has shown that MMA III increases ODD in a methylation-deficient cell line [73]. This contrasts with our data where $NaAsO_2$ did not induce ROS in primary cells from DKO mice and the methylated arsenicals did not increase ROS in the DKO mouse vessels. This could be due to the differences between primary cells, cell lines, and animals, the $apoE^{-/-}$ background, or the use of knock-out versus poor methylating cells. Despite this, our findings are intriguing and suggest that arsenic methylation is important in ROS production through a yet-to-be-identified mechanism. One

could hypothesize that: 1) The As3MT-dependent production of MMA III results in a species with higher affinity for thiols, such as GSH. If GSH is depleted more by MMA III, it would not be available to scavenge ROS; 2) Dimethyl arsenous III (DMA III) or other unknown product formed by the biotransformation reaction is driving ROS production; or 3) As3MT may have other unknown functions.

Although no functions outside of arsenic methylation have been described for As3MT, different metabolomic profiles have been observed in wild-type and As3MT$^{-/-}$ mice in the absence of arsenic [74]. In addition, a genome-wide association study investigating the schizophrenia-associated locus linked a polymorphism in the first exon to generation of an alternatively spliced As3MT isoform, which is potentially a molecular marker for this psychiatric disease [75]. Deletion of As3MT from apoE$^{-/-}$ mice mildly increased the basal plaque size at 17 weeks of age in the sinus (Figure 2.2), which was also observed in DKO mice translplanted with DKO bone marrow (Figure 2.5). A higher basal level of plaque was also observed in apoE$^{-/-}$ mice reconstituted with DKO bone marrow (Figure 2.5). These increases in basal plaque levels upon As3MT deletion were modest in comparison to high-fat diet, although thus far, we have only tested mice at this relatively early time point in atherogenesis. Nevertheless, our data suggest that As3MT may protect against atherosclerosis in the absence of arsenic. Perhaps when present, arsenic quenches or sequesters As3MT, preventing anti-atherogenic functions. This could explain both the increased basal plaque level upon As3MT deletion and the lack of arsenic-enhanced atherosclerosis in the DKO.

In summary, our data suggest that As3MT is required for arsenic-enhanced atherosclerosis, although the molecular mechanisms involved and the interaction with arsenic remain to be elucidated. As3MT SNPs may be useful biomarkers for identifying those at greatest risk for developing cardiovascular toxicity associated with arsenic exposure.

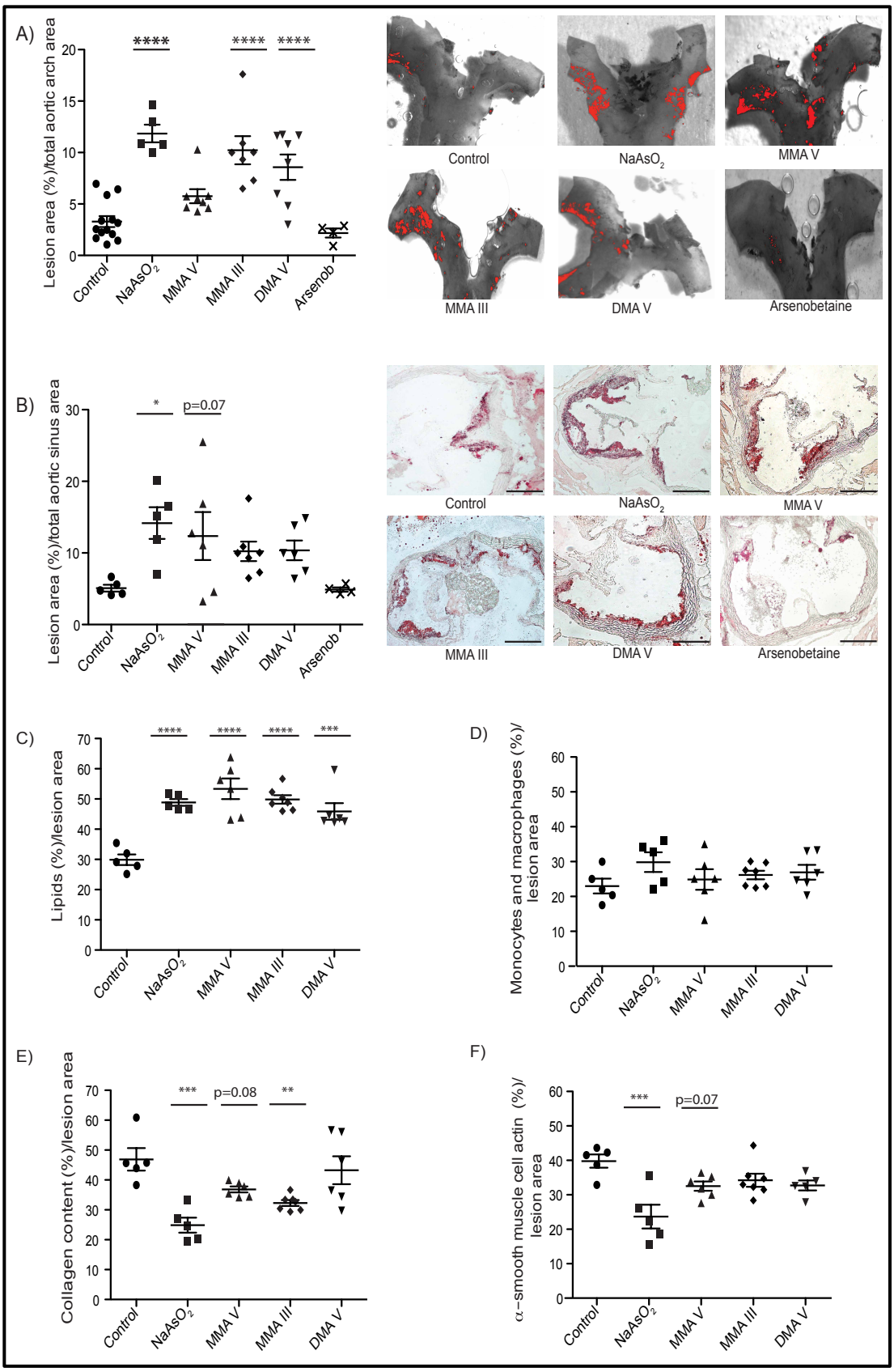

A)
Lesion area (%)/total aortic arch area
****
****
****
Control
NaAsO2
MMA V
MMA III
DMA V
Arsenob
Control
NaAsO2
MMA V
MMA III
DMA V
Arsenobetaine
B)
Lesion area (%)/total aortic sinus area
*
p=0.07
Control
NaAsO2
MMA V
MMA III
DMA V
Arsenob
Control
NaAsO2
MMA V
MMA III
DMA V
Arsenobetaine
C)
Lipids (%)/lesion area
****
****
****
***
Control
NaAsO2
MMA V
MMA III
DMA V
D)
Monocytes and macrophages (%)/ lesion area
Control
NaAsO2
MMA V
MMA III
DMA V
E)
Collagen content (%)/lesion area
***
p=0.08
**
Control
NaAsO2
MMA V
MMA III
DMA V
F)
α–smooth muscle cell actin (%)/ lesion area
***
p=0.07
Control
NaAsO2
MMA V
MMA III
DMA V

Figure 2.1: Methylated arsenicals increase plaque formation and alter plaque components.

Four week old apoE$^{-/-}$ mice were exposed to 200 ppb arsenicals [$NaAsO_2$, MMA V, MMA III, DMA V or arsenobetaine (arsenob)] or maintained on tap water for 13 weeks. Percent lesion area of the aortic arch (A) or aortic sinus (B) was evaluated via oil red O. Lipid content (C), macrophage (D), collagen (E), and smooth muscle cell (F) content was evaluated in the aortic sinus relative to the total lesion area. Scale bar represents 100 μm. Values are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ relative to their own control.

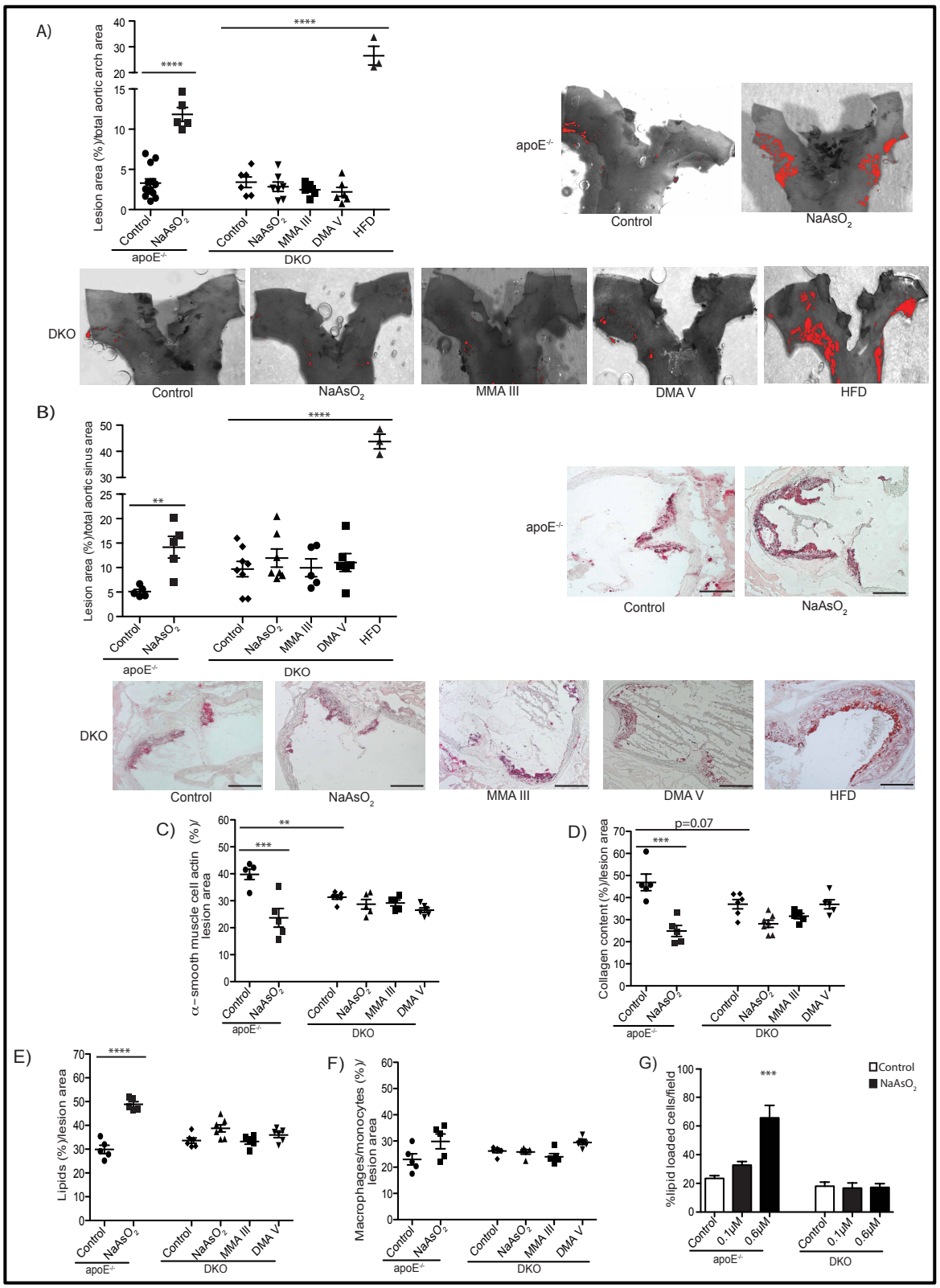

A)
Lesion area (%)/total aortic arch area
****
****
Control
NaAsO2
MMA III
DMA V
HFD
apoE-/-
DKO
apoE-/-
Control
NaAsO2
DKO
Control
NaAsO2
MMA III
DMA V
HFD
B)
Lesion area (%)/total aortic sinus area
****
**
Control
NaAsO2
MMA III
DMA V
HFD
apoE-/-
DKO
apoE-/-
Control
NaAsO2
DKO
Control
NaAsO2
MMA III
DMA V
HFD
C)
α-smooth muscle cell actin (%)/ lesion area
**
***
Control
NaAsO2
MMA III
DMA V
apoE-/-
DKO
D)
Collagen content (%)/lesion area
p=0.07
***
Control
NaAsO2
MMA III
DMA V
apoE-/-
DKO
E)
Lipids (%)/lesion area
****
Control
NaAsO2
MMA III
DMA V
apoE-/-
DKO
F)
Macrophages/monocytes (%)/ lesion area
Control
NaAsO2
MMA III
DMA V
apoE-/-
DKO
G)
%lipid loaded cells/field
***
Control
NaAsO2
Control
0.1μM
0.6μM
apoE-/-
Control
0.1μM
0.6μM
DKO

Figure 2.2: As3MT expression is required for arsenic-enhanced atherosclerosis and lipid accumulation on macrophages.

Four week old apoE$^{-/-}$ and DKO mice were exposed to 200 ppb arsenicals ($NaAsO_2$, MMA III or DMA V) or maintained on tap water for 13 weeks. An additional group of DKO mice was fed a high fat diet (HFD) and kept on tap water. Percent lesion area of the aortic arch (A) or aortic sinus (B) was evaluated via oil red O. Smooth muscle cell (C), collagen (D), lipid (E), and macrophage (F) content was evaluated in the aortic sinus relative to the total lesion area. Representative pictures are shown. Scale bar represents 100 μm. Bone marrow-derived macrophages from apoE$^{-/-}$ or DKO background were cultured for 5 days with or without 0.1 or 0.6 μM (10 or 50 ppb) $NaAsO_2$, after which cultures were challenged with 7-ketocholesterol for 24 hours. The number of cells staining positive for lipids is expressed relative to total cell amount per field was quantified (G). Values are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ relative to their own control.

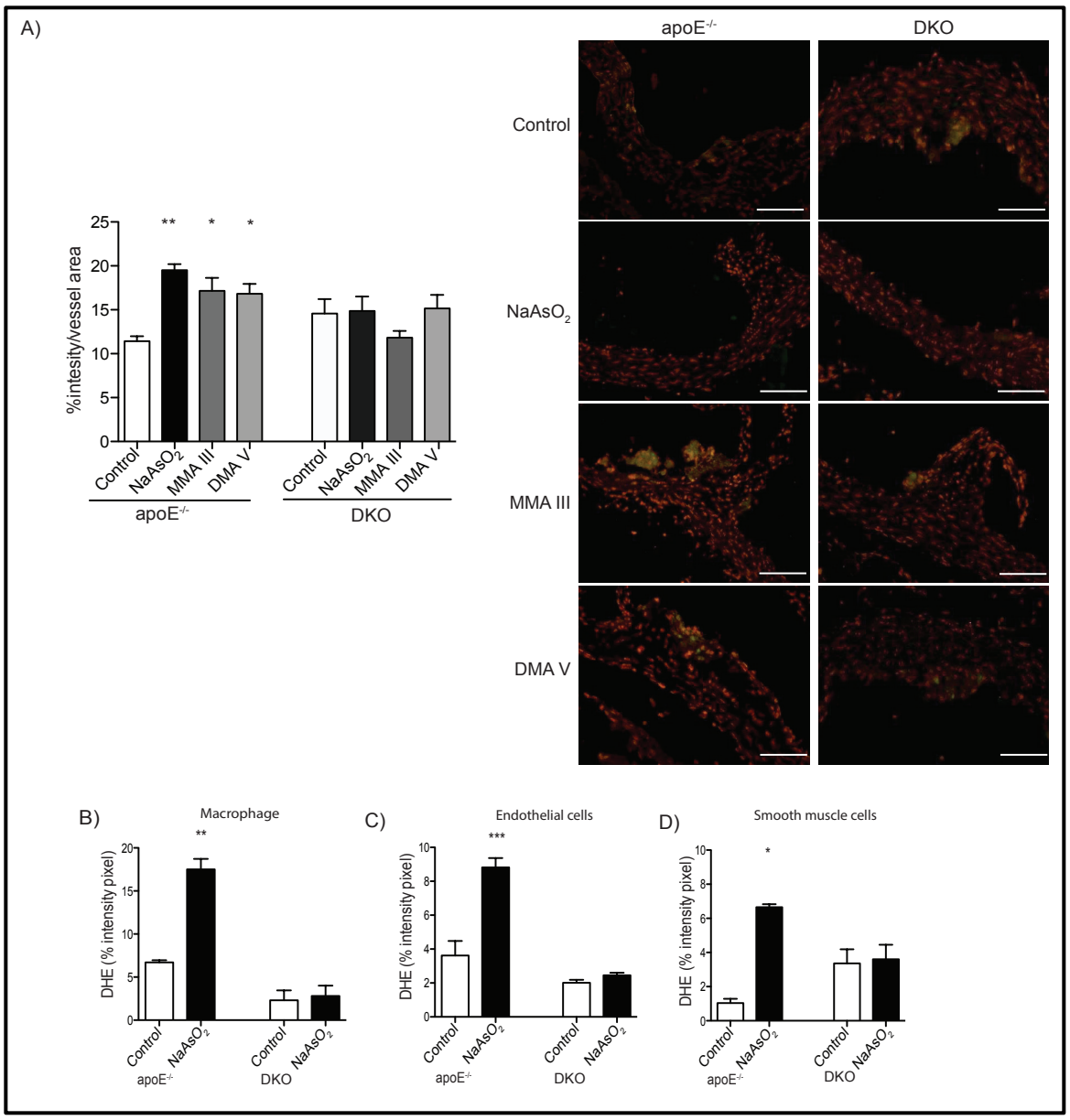

A)
apoE-/-
DKO
Control
NaAsO2
MMA III
DMA V
%intesity/vessel area
25
20
15
10
5
0
**
*
*
Control
NaAsO2
MMA III
DMA V
apoE-/-
DKO
B)
Macrophage
DHE (% intensity pixel)
**
C)
Endothelial cells
***
D)
Smooth muscle cells
*

Figure 2.3: Arsenic (3) methyltransferase is necessary for arsenic increase ROS production.

Four week old apoE$^{-/-}$ and DKO mice were exposed to arsenicals ($NaAsO_2$, MMA V, MMA III or DMA V) at 200 ppb for 13 weeks or maintained on tap water. Frozen sections (at least 3 sections per animal; n>3 per group) of the aortic sinus were stained with 2 μM dihydroethidium and ROS levels were expressed as percentage of the total vessel area (A). Representative pictures are shown. Scale bar represents 200μm. BMDM (B), endothelial cells (C) and smooth muscles cells (D) derived from apoE$^{-/-}$ or DKO mice were pre-treated with 5 μM dihydroethidium for 10 min, then cultures were exposed to 2.6 μM (200 ppb) $NaAsO_2$ for another 30 min. Data are expressed as mean ± SD from three independent experiments. *$p < 0.05$; **$p < 0.01$ relative to their own control.

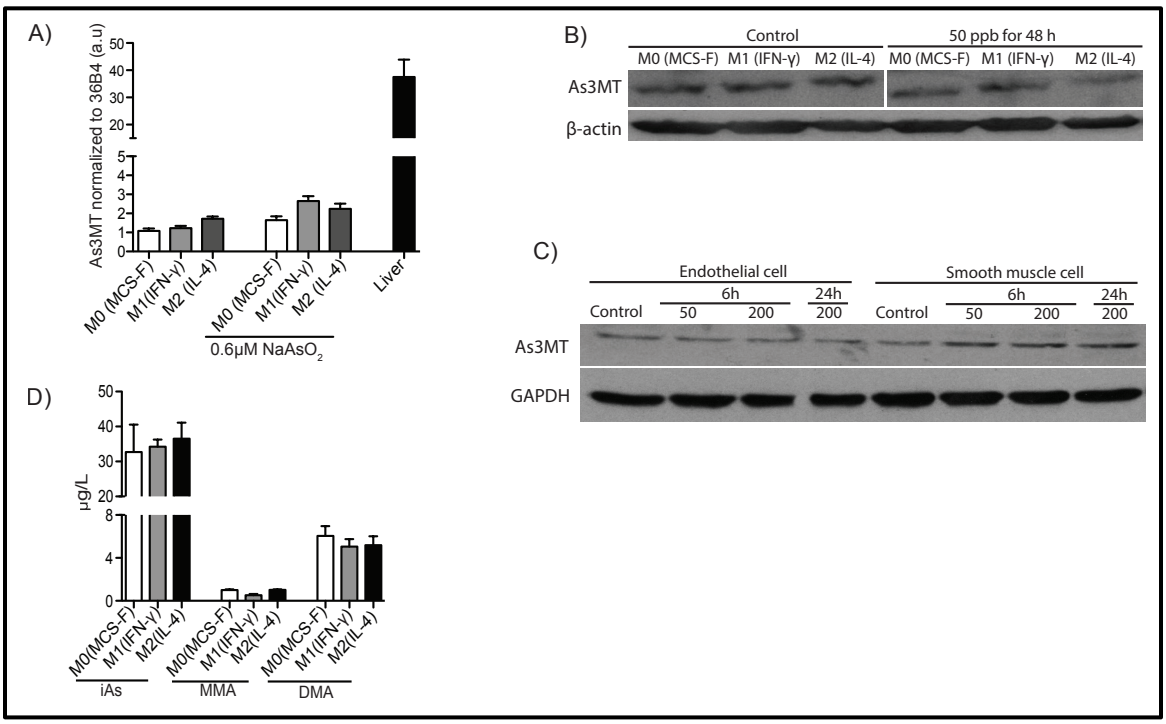

A)
As3MT normalized to 36B4 (a.u)
M0 (MCS-F)
M1(IFN-γ)
M2 (IL-4)
M0 (MCS-F)
M1(IFN-γ)
M2 (IL-4)
Liver
0.6μM NaAsO2
B)
Control
50 ppb for 48 h
M0 (MCS-F) M1 (IFN-γ) M2 (IL-4)
M0 (MCS-F) M1 (IFN-γ) M2 (IL-4)
As3MT
β-actin
C)
Endothelial cell
Smooth muscle cell
Control
6h
24h
50
200
200
Control
6h
24h
50
200
200
As3MT
GAPDH
D)
μg/L
M0(MCS-F)
M1(IFN-γ)
M2(IL-4)
M0(MCS-F)
M1(IFN-γ)
M2(IL-4)
M0(MCS-F)
M1(IFN-γ)
M2(IL-4)
iAs
MMA
DMA

Figure 2.4: BMDM, endothelial cells and smooth muscle cells express functional As3MT.

Total RNA from BMDM and liver from apoE$^{-/-}$ mice was isolated and As3MT gene expression was assessed by qPCR. Each sample was analyzed in triplicate (technical replicate) and expressed relative to the m36B4 housekeeping gene (arbitrary units) (A). Immunoblots for As3MT were performed from whole cell extracts of BMDM (B), endothelial cells or SMC cultures (C) exposed to $NaAsO_2$ at the time and concentration as indicated. Three independent experiments were performed. Arsenic methylation profiles in the media from BMDM (D) were assessed by HPLC-ICP-MS, iAs- inorganic arsenic, MMA – monomethyl arsenic, DMA – dimethyl arsenic.

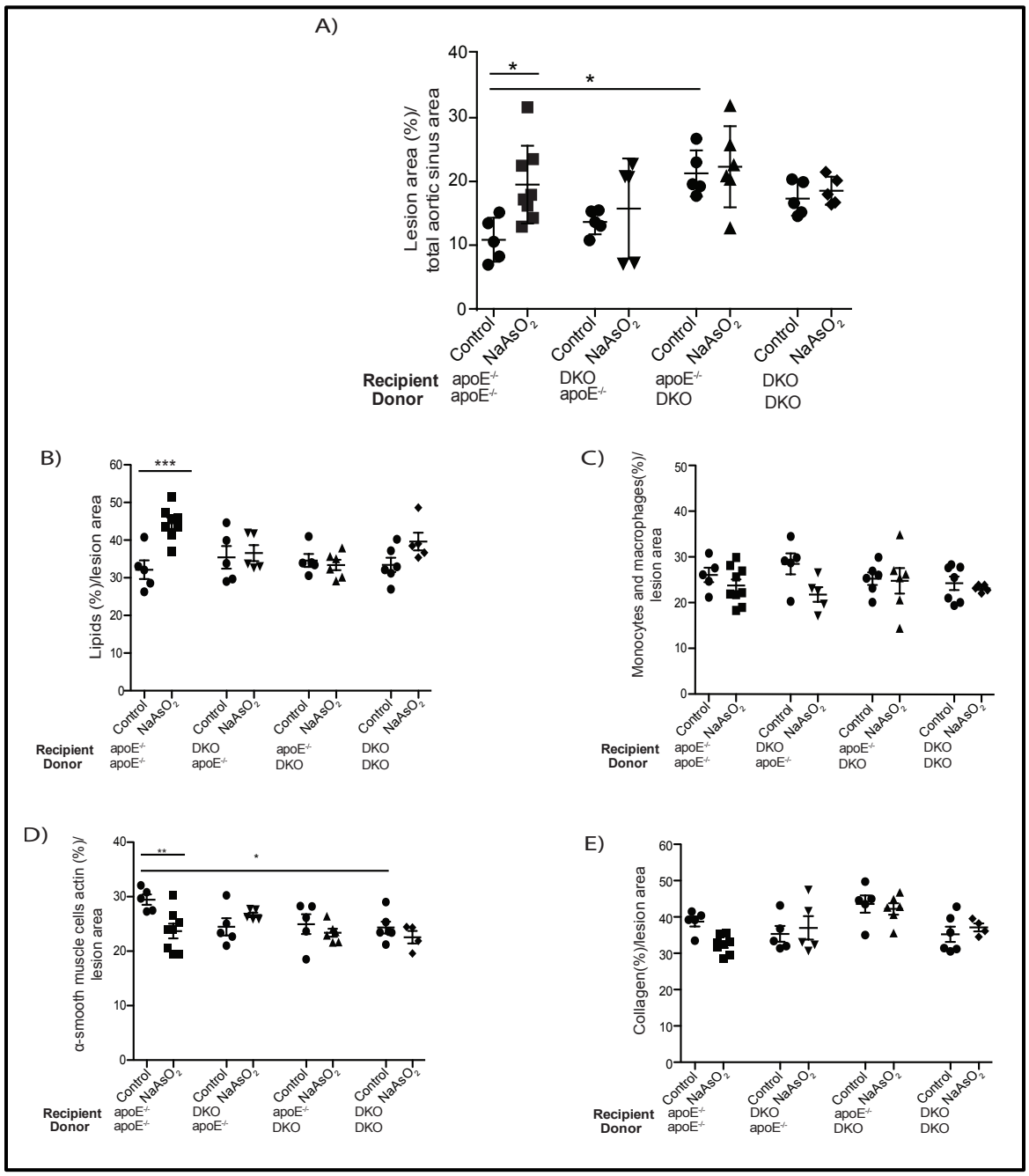
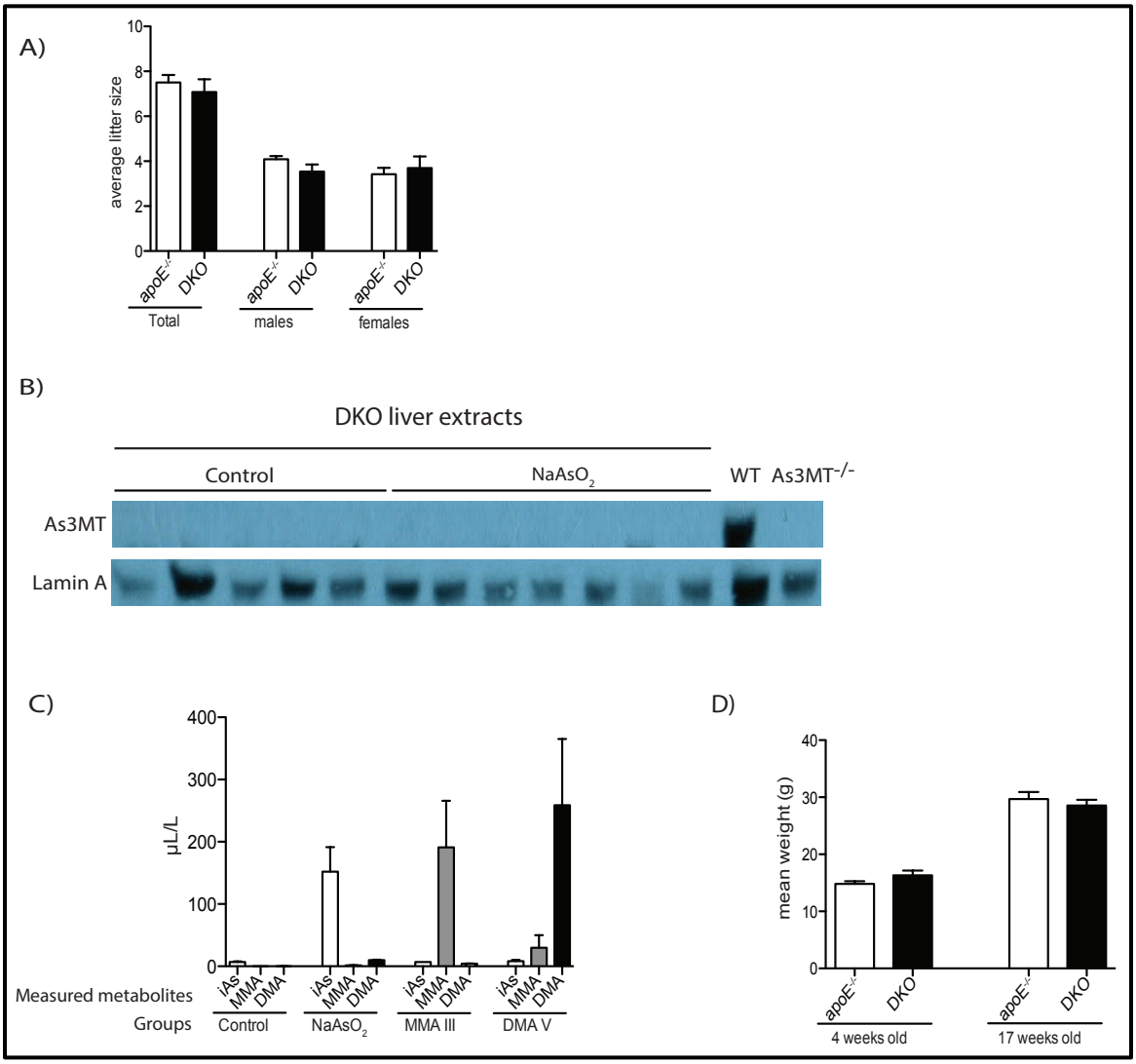

A)
Lesion area (%)/ total aortic sinus area
*
*
Control
NaAsO2
Recipient
Donor
apoE-/-
apoE-/-
DKO
apoE-/-
apoE-/-
DKO
DKO
DKO
B)
Lipids (%)/lesion area
***
C)
Monocytes and macrophages(%)/ lesion area
D)
α-smooth muscle cells actin (%)/ lesion area
**
*
E)
Collagen(%)/lesion area

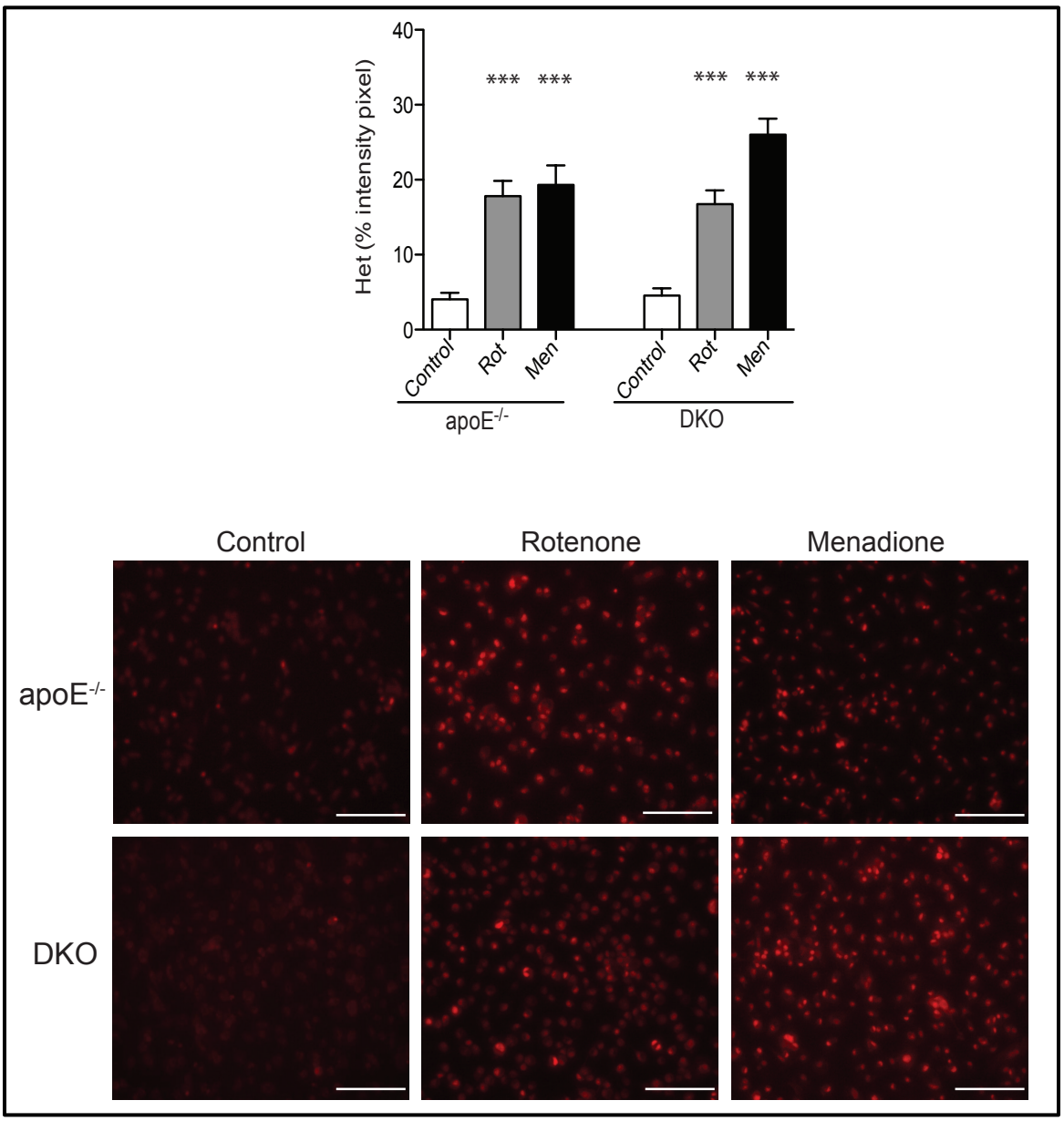
Figure 2.5: Bone marrow transplantation studies show a role for As3MT in both bone marrow-derived and host cells in arsenic-enhanced atherosclerosis

Five week old apoE$^{-/-}$ or DKO male mice were lethally irradiated and transplanted as indicate. Transplanted nine week old apoE$^{-/-}$ mice with apoE$^{-/-}$ BM, DKO mice with apoE$^{-/-}$ BM, apoE$^{-/-}$ mice with DKO BM, DKO mice with DKO BM were exposed to $NaAsO_2$, at 200 ppb for 13 weeks or maintained on tap water. Sections of the aortic sinus were stained with oil red O, and percentage of the lesion area was evaluated relative to the total aortic sinus area (B), the lipid content was evaluated relative to the total lesion area (C). Scale bars 100 μM. Sections were stained for macrophages composition (D). Values are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ relative to their own control.


A)
average litter size
10
8
6
4
2
0
apoE-/-
DKO
Total
males
females
B)
DKO liver extracts
Control
NaAsO2
WT
As3MT-/-
As3MT
Lamin A
C)
µL/L
400
300
200
100
0
iAs
MMA
DMA
Measured metabolites
Groups
Control
NaAsO2
MMA III
DMA V
D)
mean weight (g)
40
30
20
10
0
4 weeks old
17 weeks old

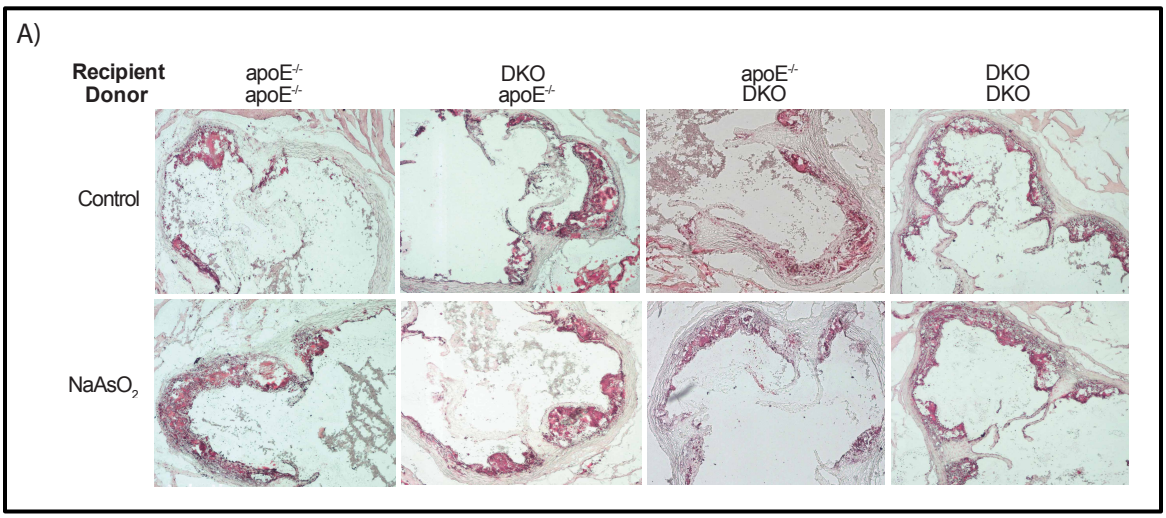
Supplementary Figure 2.1: DKO model characterization and validation

The litter size and sex ratio was similar in at least 12 mating pairs of apoE-/- and DKO mice (A). As3MT immunoblot of liver extracts from DKO mice (control and NaAsO2-exposed), wild-type C57BL6 and As3MT$^{-/-}$ mice (B). Results from arsenic speciation by HPLC/ICP-MS in urine samples from DKO mice exposed to NaAsO2, MMA III, DMA V, or tap water (C). DKO mice were unable to significantly metabolize arsenicals. Unexposed mice from both genotypes had similar weight at 4 weeks and 17 weeks (D).


Het (% intensity pixel)
40
30
20
10
0
*** ***
*** ***
Control
Rot
Men
Control
Rot
Men
apoE-/-
DKO
Control
Rotenone
Menadione
apoE-/-
DKO

Supplementary Figure 2.2: apoE$^{-/-}$ and DKO macrophages are responsive to reactive oxygen induction

Bone marrow-derived macrophages from apoE$^{-/-}$ or DKO mice from three independent experiments, were pre-treated with 5μM of dihydroethidium for 10 min, then cultures were exposed to 10 μM rotenone (Rot) or 200μM menadione (Men) for a further 30 min. Cell were imaged and representative pictures are shown.


A)
Recipient
Donor
apoE-/-
apoE-/-
DKO
apoE-/-
apoE-/-
DKO
DKO
Control
NaAsO2

Supplementary Figure 2.3: Representative pictures from aortic sinus of transplanted groups exposed to NaAsO2 or tap water.

Five week old apoE$^{-/-}$ or DKO male mice were lethally irradiated and transplanted as indicated in the text. Transplanted nine week old apoE$^{-/-}$ mice with apoE$^{-/-}$ BM, DKO mice with apoE$^{-/-}$ BM, apoE$^{-/-}$ mice with DKO BM, DKO mice with DKO BM were exposed to NaAsO2, at 200 ppb for 13 weeks or maintained on tap water. Sections of the aortic sinus were stained with oil red O.

Supplementary Table 2.1: Effect of arsenicals on plasma cholesterol, triglycerides, HDL, LDL, ALT and AST levels of apoE$^{-/-}$ and DKO mice.

Groups	Cholesterol mmol/L	Triglycerides mmol/L	HDL mmol/L	LDL mmol/L	ALT U/L	AST U/L
Control	11.4 ± 2.2	1.2 ± 0.2	1.7 ± 0.2	9.1 ± 2.0	56 ± 19	129 ± 32
$NaAsO_2$	15.3 ± 3.1	1.5 ± 0.7	1.6 ± 0.2	12.8 ± 3.0	54 ± 06	104 ± 03
MMA V	14.1 ± 1.0	1.4 ± 0.4	1.9 ± 0.1	11.4 ± 1.1	59 ± 21	132 ± 67
MMA III	11.0 ± 3.5	1.3 ± 0.1	2.1 ± 0.1	8.7 ± 2.8	62 ± 13	177 ± 48
DMA V	13.7 ± 1.4	1.4 ± 0.4	2.1 ± 0.3	10.9 ± 1.3	57 ± 16	122 ± 32
DKO Control	14.4 ± 5.1	1.3 ± 0.2	1.8 ± 0.3	11.7 ± 5.5	68 ± 34	128 ± 56
DKO $NaAsO_2$	15.7 ± 1.8	1.7 ± 0.2	2.1 ± 0.3	12.7 ± 1.6	63 ± 13	131 ± 24

No statistical difference was observed

Supplementary Table 2.2: Complete blood count from control apoE$^{-/-}$ and DKO mice.

	apoE$^{-/-}$	**DKO**
RBC (10^6/mm^3)	10.1 ± 0.6	11.4 ± 2.2
HGB (g/dL)	16.1 ± 0.9	17.8 ± 2.8
HCT (%)	48.4 ± 2.6	48.4 ± 2.6
MCV (μm^3)	48.2 ± 1.1	47.5 ± 1.0
MCH (pg)	16.0 ± 0.4	15.6 ± 0.8
MCHC (g/dL)	33.3 ± 0.5	32.9 ± 1.0
RDW (%)	15.2 ± 0.9	15.1 ± 0.9
WBC (10^3/mm^3)	7.6 ± 2.9	6.5 ± 1.4
Lymphocyte (10^3/mm^3)	3.2 ± 1.0	3.1 ± 0.7
Monocytes (10^3/mm^3)	0.6 ± 0.2	0.6 ± 0.1
Granulocytes (10^3/mm^3)	3.9 ± 1.8	2.9 ± 0.7

No statistical differences were observed

2.6 References

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Preface

We have shown for the first time that methylated arsenicals are atherogenic and that As3MT expression is required for arsenic-enhanced atherosclerosis. However, arsenic exposure scenarios are much more diverse. For example, people may live in high arsenic area and then move to a non-endemic region, therefore subjects can be exposed only during development.. Moreover, there is a necessity to better understand later in life health effects of arsenic after exposure during pregnancy. Thus, we were interested to assess, in an environmental relevant concentration, the potential of arsenicals to enhance atherosclerosis later in life after *in utero* exposure.

CHAPTER 3

Prenatal arsenic exposure enhances atherosclerosis via an As3MT-dependent mechanism

This work is in preparation for publication as following:

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3.1 Abstract.

Arsenic is a toxicant naturally found in soil, water and air, to which millions of people are exposed worldwide. Pregnancy and development are considered a susceptible window of exposure. Thus, we were interested to assess the potential of arsenicals, at an environmentally-relevant concentration, to enhance atherosclerosis later in life after *in utero* exposure. Using a similar mouse model, we exposed mice from the conception to weaning to arsenicals or tap water and assessed the extent of atherosclerosis after another 13 weeks in the absence of arsenic. Surprisingly, sodium arsenite and methylated arsenical exposure was associated with an increase in plaque formation in the aortic arch and aortic sinus in males and females. In this model, reactive oxygen species were not induced. In addition, we found that in the absence of As3MT, arsenicals failed to enhance plaque formation after *in utero* sodium arsenite exposure. Therefore, our data show that prenatal exposure to inorganic arsenic and methylated-arsenicals have the potential to increase atherosclerosis, which is dependent on As3MT expression

3.2 Introduction.

Arsenic is a toxicant naturally found in soil, water and air, to which millions of people are exposed worldwide [1, 2]. Arsenic has been identified to increase the risk of several diseases, from cancer to cardiovascular disease [3-7]. Our lab has shown in an animal model that arsenic increases the formation of atherosclerosis [8], through mechanisms that include enhanced accumulation of lipids in macrophages [9] and increased attachment of macrophages to the arterial endothelial cell layer [10].

Arsenic is metabolized by a series of oxidative methylation reactions catalyzed by the enzyme arsenic (III) methyltransferase (As3MT) [11]. The biotransformation process is a conserved mechanism that produces methylated intermediate compounds, and has historically been considered a detoxification process. However, this detoxification concept has been challenged, because some of the intermediate compounds are considered more toxic than the inorganic form [12]. For example, we have shown that methylated forms are as pro-atherogenic as inorganic arsenic [13]. Interestingly, we also showed that arsenic-enhanced plaque formation was dependent upon As3MT expression [13]. Thus, arsenic methylation is essential for the atherogenic properties of arsenic after postnatal exposure.

Several factors may contribute to the efficiency of the biotransformation reaction, such as nutritional status [14-16], genetic background [17, 18], gender [19, 20] and pregnancy [21]. Indeed, pregnant women have a more efficient methylation process [16, 21]. Arsenic and methylated intermediates pass through the placenta, thus the fetus is exposed to similar concentrations of arsenic as the mother [16, 21]. Pregnancy and development are considered susceptible periods of exposure to toxicants [22], but the sequelae from exposure to arsenic during gestation have not been well-defined.

In utero exposure to arsenic is linked to effects on children, including decreased birth weight, potentially by decreasing gestational age and/or maternal weight gain [23]. A limited number of epidemiological studies suggest that *in utero* arsenic exposure is also associated with several adverse health effects later in life, such lung and bladder tumors, bronchiectasis and myocardial infarction [7, 24], [25] [26]. Limited data implicate early-life arsenic exposures to increased atherosclerosis. Increased carotid intima thickness (cIMT), a marker for early atherosclerosis, was found in a cross sectional study of children in Mexico to be associated with total urinary arsenic [27]. Although the contribution of *in utero* exposure could not be defined, most of the mothers lived in the same region throughout pregnancy. In a

Bangladeshi cohort, arsenic exposure was associated with greater risk of mortality from cancer or CVD in children, albeit in a sample population with a very small death rate [28]. Thus, it is unclear whether exposure to arsenic during gestation leads to increased risk of atherosclerosis in humans.

Animal experiments have been useful in identifying the underlying mechanisms of arsenic enhanced atherosclerosis later in life. Indeed, exposure to inorganic arsenic increased plaque formation in apoE knockout mice exposed *in utero* [29]. Microarray analysis showed that preprograming of oxidative stress and inflammatory response pathways was associated with enhanced development of atherosclerosis later in life. Lipids, stress and inflammatory pathways, which may contribute to plaque formation, were also up-regulated after arsenical exposure [30]. One limitation of these studies was the high concentration of arsenic used (49 ppm). Thus, it is unknown whether prenatal exposure to low to moderate arsenic concentrations are pro-atherogenic. Additionally, the pro-atherogenic potential of methylated arsenicals and the impact of biotransformation remain to be elucidated.

Thus, we asked whether prenatal exposure to moderate concentrations of inorganic and methylated arsenicals would increase atherosclerosis when measured later in life. Furthermore, we utilized our model of $ApoE^{-/-}$ mice that also lack As3MT expression to determine whether arsenic-enhanced atherosclerosis following gestational exposure was dependent upon As3MT.

3.3 Material and Methods.

3.3.1 Mice and exposure protocol.

B6.129P2-*apoE*^{tm1Unc}/J (apoE$^{-/-}$) mice were obtained from Jackson laboratory (Bar Harbor, ME, USA). *As3MT*$^{-/-}$ mice (C57BL/6 background) were kindly provided by David Thomas (US Environmental Protection Agency, Research Triangle Park, NC, USA). ApoE$^{-/-}$As3MT$^{-/-}$ double knockout [31] mice were created in our facility [13]. Purchased mice were acclimatized to housing conditions under a 12-hour light/12-hour dark cycle for at least 2 weeks before experiments. All mice were fed *ad libitum*. The experimental protocol was approved by the McGill Animal Care Committee and animals were handled in accordance with institutional guidelines. McGill Animal Care Committee is certified by the Canadian Council on Animal Care.

Mice were assigned randomly by sex to mating pairs (n=3-4 pairs per experimental condition). Mating pairs were exposed to tap water or tap water containing 200 ppb *m*-sodium arsenite (0.35 mg/L $NaAsO_2$; Sigma-Aldrich, Ontario, Canada), disodium methyl arsonate hexahydrate DSMA (MMA V; 0.78 mg/L; Chem Service, West Chester, PA USA), monomethyl arsenous acid [MMA III; 0.37 mg/L, synthesized as described [32]] or cacodylic acid (DMA V; 0.43 mg/L; Sigma-Aldrich, Saint Louis, MO USA). Solutions containing arsenic were refreshed every 2-3 days to minimize oxidation. After confirmation of pregnancy, males were removed from cages. Exposure was from conception until weaning (3 weeks after birth). At 4 weeks, apoE$^{-/-}$ or DKO were maintained for an additional 13 weeks on tap water. Male and female offspring were kept for plaque formation assessment and analysis. The mice were fed AIN-76A purified diet (Harlan Laboratories Inc, WI, USA) containing 5% fat (by weight) with no cholesterol for all the experiments.

3.3.2 Plasma analyses.

Blood (0.6 ml) was collected by cardiac puncture and plasma was obtained using collection tubes (EDTA BD Vacutainer SST, USA). Cholesterol, high (HDL) and low-density (LDL) lipoproteins, triglycerides and liver enzymes [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] were assessed by the Animal Resources Centre (McGill University, Canada) (Supplementary Table 1 and Table 2).

3.3.3 Atherosclerotic lesion characterization.

The characterization of the atherosclerotic lesions was performed as previously described [8]. Briefly, the fixed aorta was rinsed with ultra pure water, then cut longitudinally and stained *en face* with oil red O (Electronic Microscopy Sciences). Images were acquired using *Infinity Capture* software and camera (Lumenera, Canada). Percentage of lesion area of the aortic arch, as defined as the region from the first intercostal arteries to the ascending arch, was evaluated with *Image J* software (National Institute of Health, USA; NIH). The atherosclerotic lesions were also evaluated within the aortic sinus from at least 6 animals. Rinsed, fixed and embedded frozen hearts were processed as previously described [8]. Consecutive, 6 µm cryosections were sliced from the aortic base throughout the aortic sinus, where three to five valve sections per animal were stained with oil red O to visualize the plaque areas and were analyzed for their lipid content. Aortic valves were also stained and analyzed for their collagen content (type I and III) using picrosirius red (Polysciences, PA, USA) [8].

3.3.4 *In situ* immunofluorescence.

Smooth muscle cell (SMC) and macrophage content were assessed within the entire plaque area, as previously described [8]. Briefly, slides were rinsed with PBS, blocked with 3% BSA and incubated for 1 hour with primary antibody (1:200 for monoclonal anti-α-smooth muscle cell actin [clone 1A4], 1:50 for moma-2 [Abcam, MA, USA]), rinsed and incubated with fluorescently-labelled secondary antibodies (1:500) (Invitrogen, ON, Canada). The presence of the immunofluorescent marker from 3 to 5 sections per animal was quantified using *Image J* software and expressed as percentage of the total lesion area.

3.3.5 *In situ* dihydroethidium staining.

Frozen aortic sinus sections were stained with 2 µM dihydroethidium (ThermoFisher Scientific) in PBS and analyzed immediately using a fluorescent microscope. The presence of the fluorescent marker from 3 to 5 sections per animal was quantified using *Image J* software (NIH) and expressed as percentage of the total vessel area.

3.3.6 Statistical considerations.

Standard statistical analyses in which the assumption of independence of each of the mice cannot be used. The issue is that the mice in the same litter are more similar to each other than mice from other mothers; this dependency violates the usual assumption of independence in standard statistical models (e.g., t-tests, ordinary regression) and thus, these correlations needed to be captured in the statistical model. As well, assuming independence leads to underestimates of standard errors and consequently artificially reduced confidence intervals (CI) and p-values. Fortunately, the mixed model framework allows for these within-dam correlations and produces appropriate inferences. Thus, to meet the objectives of this study, namely to estimate the effect of arsenic on plaque size in the aortic arch and sinus and plaque contents: macrophages, lipids, collagen and SMC, we developed simple mixed regression models in which each of the exposure groups were compared to the unexposed group, for each of the outcomes separately. The mixed model framework accounted for the within-litter correlations by specifying that all mice in the same litter were grouped together (in technical terms, a random effect was placed on the intercept for each dam), regardless of which exposure group they were in. In addition, as we found that gender was associated with each outcome, and thus, this was also included in the model as a covariate. Effectively, this somewhat more complicated model is equivalent to standard t-tests comparing groups or regression models, but accounts for the within-dam correlations, thereby providing unbiased estimates of standard errors, CIs, and p-values. We verified routinely the assumptions of normality of each outcome and we used standard diagnostics to ensure that the assumptions of the model were met. The analyses were conducted in R using the lme function.

3.4 Results.

3.4.1 *In utero* arsenical exposure increases plaque formation later in life.

We recently showed that low to moderate concentrations of sodium arsenite and methylated arsenicals increase plaque formation in the apoE knockout mice model [13]. To our knowledge, the prenatal effects of methylated arsenicals on the development of atherosclerosis have never been reported. Thus, 3-4 mating pairs and subsequent litters of $apoE^{-/-}$ mice were exposed from conception to weaning to 200 ppb methylated arsenicals (MMA V, MMA III, or DMA V), inorganic arsenic ($NaAsO_2$) or tap water. After weaning, all pups were kept on tap water for an additional 13 weeks, at time point at which post-natal exposure enhances lesion formation. A description of litter number, size, and sex distribution is included in Table 1. We observed no differences in litter size or sex distribution between exposure groups.

After 13 weeks of tap water, we assessed atherosclerotic plaque size in the aortic arch (*en face)* and in the aortic sinus after staining with Oil Red O. As described previously [33, 34], females had larger plaques than males independent of arsenic exposure (Figure 3.1A and Supplementary Table 3.3), thus, we included sex in our mixed effects model as a co-variate. In addition, we observed that both inorganic and methylated intermediates increased mean plaque size in the aortic arch and aortic sinus (Figure 3.1 and Table 3.2). Inorganic arsenic (200 ppb) increased aortic arch plaque by 2.8-fold and sinus plaque by 5.3-fold, results that are similar to what was published for 49 ppm arsenic *in utero* exposure [29]. Surprisingly, methylated arsenical exposures were significantly associated with enhanced size of the aortic arch lesions (Figure 3.1A and Table 3.2). We measured circulating lipid and liver enzymes levels from the plasma of both male and female mice, and no differences were observed between control and arsenical-exposed mice (Supplementary Table 3.1 and 3.2). Together, these data highlight that moderate concentrations of inorganic and methylated arsenicals increase plaque formation later in life after prenatal exposure.

3.4.2 Arsenicals exposure *in utero* alters plaque components.

We have observed *in vitro* [9] and after post-natal exposure [8] [13] that arsenic impairs lipid handling in macrophages. Each individual macrophage retains more lipids, because of diminished cholesterol efflux [35]. Thus, we assessed both macrophage and lipid content in the plaques following *in utero* arsenic exposure. No relationship between macrophage content and arsenical exposure was observed (Figure 3.2A and Table 3.3). This indicates that in utero exposure to arsenic does not change the number of macrophages in the plaques, consistent with what we have reported after postnatal exposure to arsenicals [13]. Importantly, inorganic and methylated arsenicals increased the mean lipid content within the plaque by 2.7-9.8-fold (Figure 3.2B and Table 3.3). Interestingly, the greatest increase was observed for DMA V-exposed group (9.8, CI 95%: 4.5; 15.1).

Smooth muscle cells (SMCs) and collagen are also components of the plaque, which promote plaque stability [36]. We previously reported that postnatal arsenical exposure decreased SMCs and collagen, characteristic of plaques more prone to rupture [36] [37]. Therefore, we also investigated collagen and SMC content using histological techniques. The mean change in smooth muscle cells was decreased in all arsenical-exposed groups (decreased 3.7-6.6-fold), where the biggest reductions were observed in sodium arsenite- and DMA V-exposed groups (Figure 3.2C and Table 3.3). No consistent changes were observed in collagen content amongst exposure groups (Figure 3.2D and Table 3.3), although females had increased collagen in comparison to males (Supplementary Table 3.3). These findings suggest that prenatal exposure to arsenicals might lead to an unstable phenotype within the plaques, although the phenotype was not as profound as we have described after postnatal exposure to arsenicals.

3.4.3 Reactive oxygen species (ROS) are not induced.

Arsenic is well described in the literature as a potent inducer of ROS by several mechanisms [38] [39-41]. Reactive oxygen species are also reported to play a role in the development of atherosclerosis, and are increased in plaques from arsenic-exposed mice [42] [13]. Thus, we investigated ROS production after prenatal arsenical exposure using dihydroethidium staining. In contrast to post-natal exposures, neither male nor female mice had increased levels of ROS in the aortic sinus following inorganic or

methylated arsenical exposure in utero (Figure 3.3). Therefore, there was not persistent high level of ROS later in life.

3.4.4 As3MT knockout prevents arsenic-enhanced atherosclerosis later in life.

As3MT expression is required for arsenic-enhanced atherosclerosis after post-natal exposure [13]. Thus, we investigated the impact of the biotransformation process on atherosclerotic plaques after in utero exposure. We utilized our As3MT/apoE double knockout [31] mice, which have proven useful to study the effects of arsenic methylation in arsenic-induced atherosclerosis [13]. DKO mice were exposed from conception to weaning to 200 ppb sodium arsenite or tap water. Atherosclerotic plaque was assessed after 13 weeks on tap water. Females still had larger plaques than males when As3MT was deleted (Supplementary Table 3.4). Interestingly, sodium arsenite increased the lesion area in the aortic arch (although not statistically significant) in DKO mice, while decreasing slightly the plaque size in the sinus (Figure 3.4 and Table 3.4). Together, these results suggest that arsenic methylation may also play a role in atherosclerosis following prenatal arsenic exposure, although the findings are inconsistent.

3.5 Discussion.

Arsenic is an environmental toxicant to which millions of people are exposed to worldwide [43]. Previously, we and others have shown that post-natal exposure to both inorganic [8, 35] [44, 45] and methylated arsenicals [13] is pro-atherogenic. Here, we provide evidence that pre-natal exposure to moderate concentrations of these compounds also promotes atherosclerosis. In contrast to our findings with post-natal exposures, in utero arsenical exposure does not lead to increased ROS production or significant changes in cellular or extracellular matrix components of the plaque. These data compliment other studies showing that early life exposure to arsenic correlates with disease later in life [22, 26, 46].

Arsenic exposure during pregnancy increased the early onset of atherosclerosis in the apoE$^{-/-}$ mouse model [29, 30], however those studies used a very high concentration of arsenic (49 ppm). Our study shows that at moderate concentrations of not only sodium arsenite, but also methylated arsenicals, increase plaque formation later in life. This addresses one recent criticism of high arsenic concentrations used in murine transplacental models to show transgenerational effects, where 42.5-200 ppm arsenic were employed [47]. Thus, at least for atherosclerosis, moderate concentrations result in a similar disease phenotype as high concentrations.

In contrast to our postnatal finding, *in utero* arsenical exposure had mild alterations in the plaque components. This mild phenotype may infer that arsenic exposure has to be continuous to directly affect plaque composition. The may also be the explanation for the absence of reactive oxygen species induction. ROS are short-lived, very reactive molecules; thus constant exposure may be necessary to maintain persistent high levels. However, reactive oxygen species may play a role during the exposure. Indeed, women exposed to arsenic during pregnancy have higher levels of oxidative stress in the placenta [48], high proinflammatory cytokines [48] and ROS markers [49] in the cord blood plasma. Thus, while not increased later in life, arsenic-induced ROS may have been an important mediator of pro-atherogenic signals or programming in utero.

Surprisingly, our data have shown that arsenic biotransformation may impact how arsenic enhances atherosclerosis after prenatal exposure, nevertheless this relationship was not totally clear. Arsenic biotransformation has recently received attention in regards of its potential in arsenic pathogenesis [6, 50-52]. We were the first group to show that As3MT is required for arsenic enhanced

atherosclerosis later in life [13]. Herein, we utilized our DKO model to investigate the effects of As3MT deletion on the effects that we had observed after prenatal exposure in apoE$^{-/-}$(Figure 3.1). One hypothesis is that dimethyl arsenous III (DMA III) or other methylated intermediate may drive the effects. MMA III, MMA V and DMA V were unable to induce atherosclerosis in our post-natal exposure model in DKO mice *[13]*, but it is possible that in utero exposure could be pro-atherogenic. Incomplete arsenic methylation in humans has been associated with atherosclerosis in adults [51] and the risk of CVD [6]. However, no reports were found that associate arsenic exposure during development and/or methylation efficiency with atherosclerosis later in life.

Perhaps *in utero* exposure is altering genetic reprogramming, leaving a pro-inflammatory signature that allows the disease to develop. Indeed, upregulation of pro-inflammatory pathways was observed at 10 weeks in the liver of mice exposed prenatally to high concentrations of arsenic [30], Moreover, transcription factor binding site analysis showed that 16% of those genes had SREBP-1 binding sites. Further evaluation showed upregulation of SREBP1 in the liver, however functional analysis was not performed. Association between arsenical exposure and prenatal inflammation is also observed epidemiologically. Pathway analysis of cord blood from newborns exposed to arsenic has indicated a pro-inflammatory stimulus that leads to NF-κB signalling activation [53]. Studies reported increased inflammatory cytokines in the cord blood, oxidative stress and reduced $CD3^+$T-cell infiltration in the placenta were associated with arsenic exposure in Bangladesh [48] and increase in $CD8^+$ T cells in the cord blood of infants from US exposed to lower concentrations [54],

An increasing body of research associates epigenetic modification with arsenic exposure in cell lines [55-57] and animals models [58] most of these work were in carcinogenesis model. However no single mechanism was identified. In addition, epigenetics changes after prenatal exposure to toxicants have been suggested as the main mechanism that enhances disease later in life [59, 60]. Recently, epidemiological studies are assessing cord blood cells as a model to predict epigenetics alterations in infants. Low-level arsenic exposure has been correlated with differential methylation in CpG loci of candidate genes in the cord blood of infants from US [54]. Moreover, LINE-1 hypermethylation in leucocytes from the mothers and cord blood was associated with moderate exposure to arsenic [61]. The same group reported different patterns of methylation in different tissues after prenatal arsenic exposure:

CpG island hypermethylation in placenta, hypomethylation in umbilical artery and no alteration in human umbilical vein endothelial cells [62]. Thus, endothelial cells may not be the choice to study the later effects of arsenic; moreover our model would be ideal to study epigenetic effects, because multiple cell types could be assessed. Definitively, follow up studies would be interesting to assess whether the decreased methylation in the artery is sustainable and facilitates atherosclerosis. Interestingly, maternal urinary arsenic was associated with DNA methylation in cord blood cells, however most of the alterations were not correlated with difference in mRNA expression, thus studies should address functional analysis [63].

MicroRNA (miRNA) expression is another epigenetic modification mechanism proposed to be dysregulated upon arsenic exposure in utero. miRNA are non-coding RNA that regulate post transcriptional gene expression. miRNA upregulation in the cord blood was associated with low to moderate arsenic exposure [64]. In silico analysis related these miRNA with inhibition of innate and adaptive immune response [64].

In summary, our results show that methylated arsenicals are pro-atherogenic following prenatal exposure and that the biotransformation process is an important mechanism driving this phenotype. In addition, our data suggest that epidemiological studies should evaluate SNPs that affect arsenic methylation efficiency in relation to in utero exposure when evaluating arsenic exposed population. Finally, our data further support the need to understand the mechanism by which arsenic enhances atherosclerosis in order to define appropriate interventions, because clearly removal of arsenic is not enough to reverse damage caused by in utero exposure

Table 3.1: Characteristics of the exposed groups.

Exposure	# of litters	# of pups/litter	Males/litter
Control apoE$^{-/-}$	4	6; 6; 8; 2	3; 5; 5; 2
$NaAsO_2$ apoE$^{-/-}$	3	6; 9; 5	2; 4; 3
MMA V apoE$^{-/-}$	3	6; 7; 8	3; 2; 5
MMA III apoE$^{-/-}$	3	6; 5; 4	3; 3; 2;
DMA V apoE$^{-/-}$	3	3; 4; 7	3; 2; 3
Control DKO	4	5; 5; 4; 3	2; 2; 4; 3
$NaAsO_2$ DKO	4	4; 7; 6; 4	2; 4; 3; 0

Table 3.2: Changes in the plaque size in aortic arch and sinus of arsenical-treated apoE$^{-/-}$ mice compared to control apoE$^{-/-}$ mice.

Groups	Aortic arch (%lesion area)		Aortic Sinus (%lesion area)	
	Mean change (95% CI)	p value	Mean change (95% CI)	p value
$NaAsO_2$	2.8 (-0.0; 5.6)	0.0530	5.3 (-0.5; 11.1)	0.0693
MMA V	7.1 (4.3; 9.9)	**0.0001***	3.6 (-2.1; 9.4)	0.1906
MMA III	10.2 (7.2; 13.3)	**0.0000***	8.3 (2.3; 14.3)	**0.0110***
DMA V	5.6 (2.4; 8.8)	**0.0023***	4.7 (-1.5; 10.8)	0.1205

Results for exposure groups were adjusted for sex
CI: confidence interval

Table 3.3: Changes in the plaque components (macrophages, lipids, smooth muscle cells and collagen) in arsenical-exposed apoE$^{-/-}$ mice compared to control apoE$^{-/-}$ mice.

Groups	Macrophages		Lipids		SMC		Collagen	
	Mean change (95% CI)	**p value**	**Mean change (95% CI)**	**p value**	**Mean change (95% CI)**	**p value**	**Mean change (95% CI)**	**p value**
$NaAsO_2$	-1.8 (-.5.9; 2.2)	0.3342	3.7 (-1.2; 8.7)	0.1269	-6.2 (-11.9; -0.4)	**0.0370***	-0.7(-10.6; 9.1)	0.8720
MMA V	0.4 (-3.6; 4.4)	0.6167	3.4 (-1.5; 8.4)	0.1547	-4.3 (-10.1; 1.3)	0.1204	-2.7 (-12.5; 7.1)	0.5570
MMA III	-1.0 (-5.2; 3.2)	0.7807	2.7 (-2.5; 7.8)	0.2824	-3.7(-9.6; 2.2)	0.1986	0.2 (-9.8; 10.1)	0.9704
DMA V	0.6 (-3.7; 4.8)	0.8188	9.8 (4.5; 15.1)	**0.0016***	-6.6 (-12.6; -0.6)	**0.0327***	-6.4 (-16.6; 3.6)	0.1889

Results for exposure groups were adjusted by sex
CI: confidence interval.

Table 3.4: Changes in the plaque size at the aortic arch and sinus in DKO mice exposed to $NaAsO_2$ compared to control DKO mice.

Group	Aortic arch (%lesion area)		Aortic Sinus (%lesion area)	
	Mean change (95% CI)	p value	Mean change (95% CI)	p value
$NaAsO_2$	2.5 (-1.3; 6.5)	0.1682	-0.4 (-3.9; 3.0)	0.7823

Results for exposure groups were adjusted by sex
CI: confidence interval

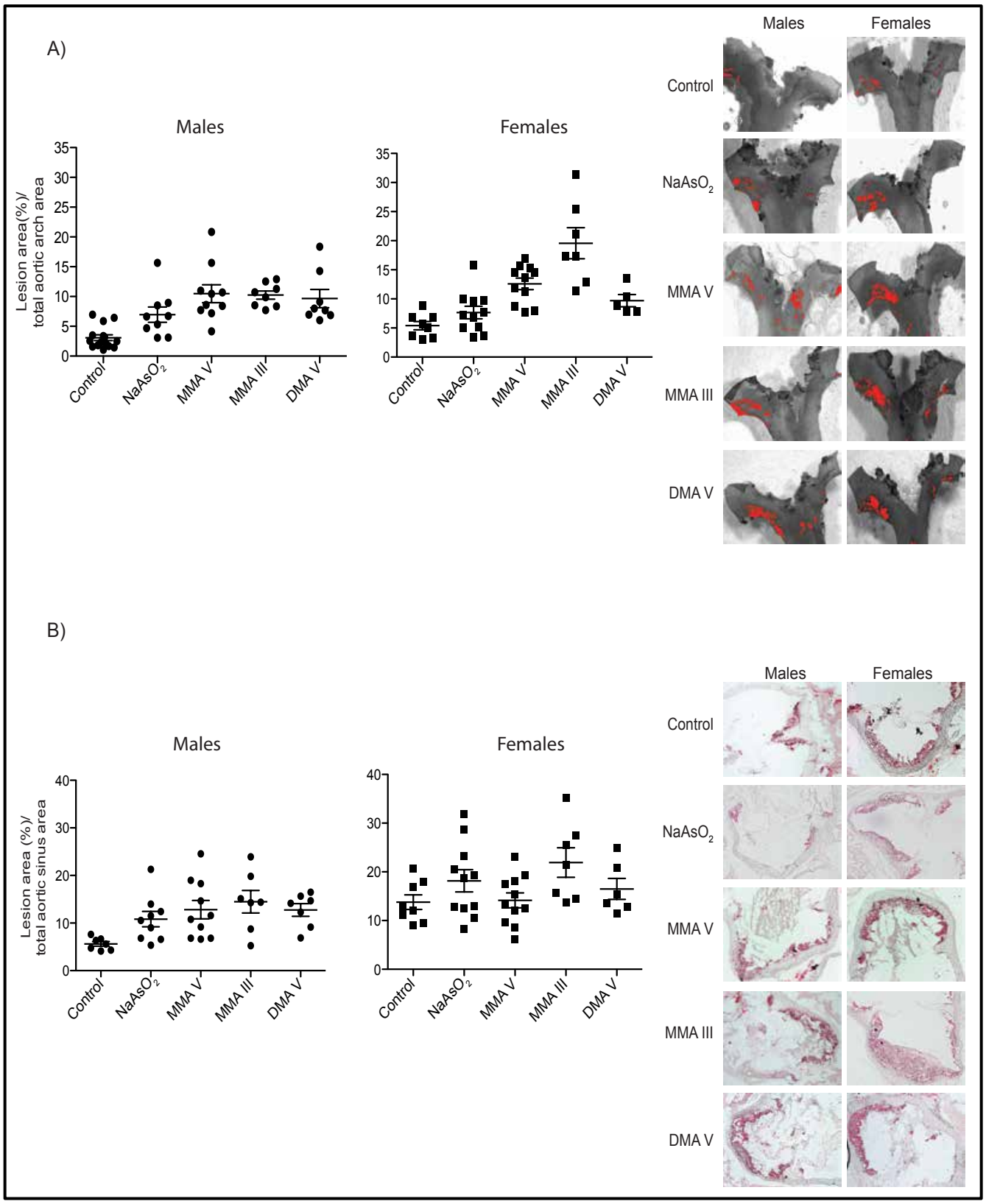
A)
Males
Females
Lesion area(%)/ total aortic arch area
Control
NaAsO2
MMA V
MMA III
DMA V
B)
Lesion area (%)/ total aortic sinus area

Figure 3.1: *In utero* exposure to sodium arsenite and methylated arsenicals increases plaque formation later in life.

ApoE$^{-/-}$ mice were exposed to 200 ppb arsenicals ($NaAsO_2$, MMA V, MMA III or DMA V) or maintained on tap water from conception to weaning. After weaning (4 weeks), male and female pups were kept on tap water for an additional 13 weeks. Percent lesion area of the aortic arch (A) or aortic sinus (B) was evaluated via oil red O staining. Values are expressed as mean ± SD.

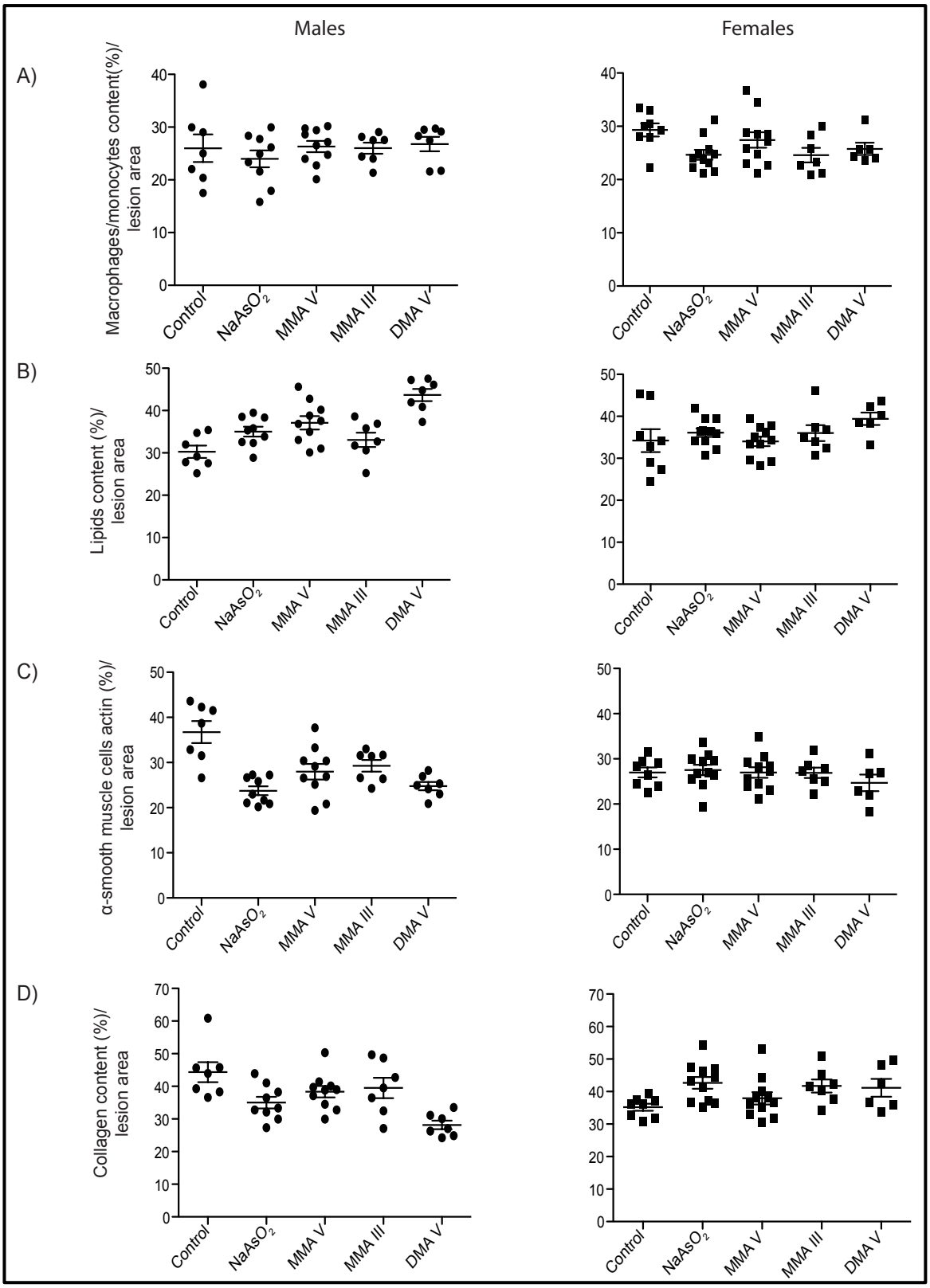
Males
Females
A)
Macrophages/monocytes content(%)/ lesion area
B)
Lipids content (%)/ lesion area
C)
α-smooth muscle cells actin (%)/ lesion area
D)
Collagen content (%)/ lesion area
Control
NaAsO2
MMA V
MMA III
DMA V

Figure 3.2: *In utero* arsenical exposure alters plaque components later in life.

ApoE$^{-/-}$ mice were exposed to 200 ppb arsenicals ($NaAsO_2$, MMA V, MMA III or DMA V) or maintained on tap water from conception to weaning. After weaning (4 weeks), male and female pups were kept on tap water for an additional 13 weeks. Macrophage (A), lipid (B), smooth muscle cell (C), and collagen (D) content were evaluated in the aortic sinus relative to the total lesion area. Scale bar represents 100 μm. Values are expressed as mean ± SD.

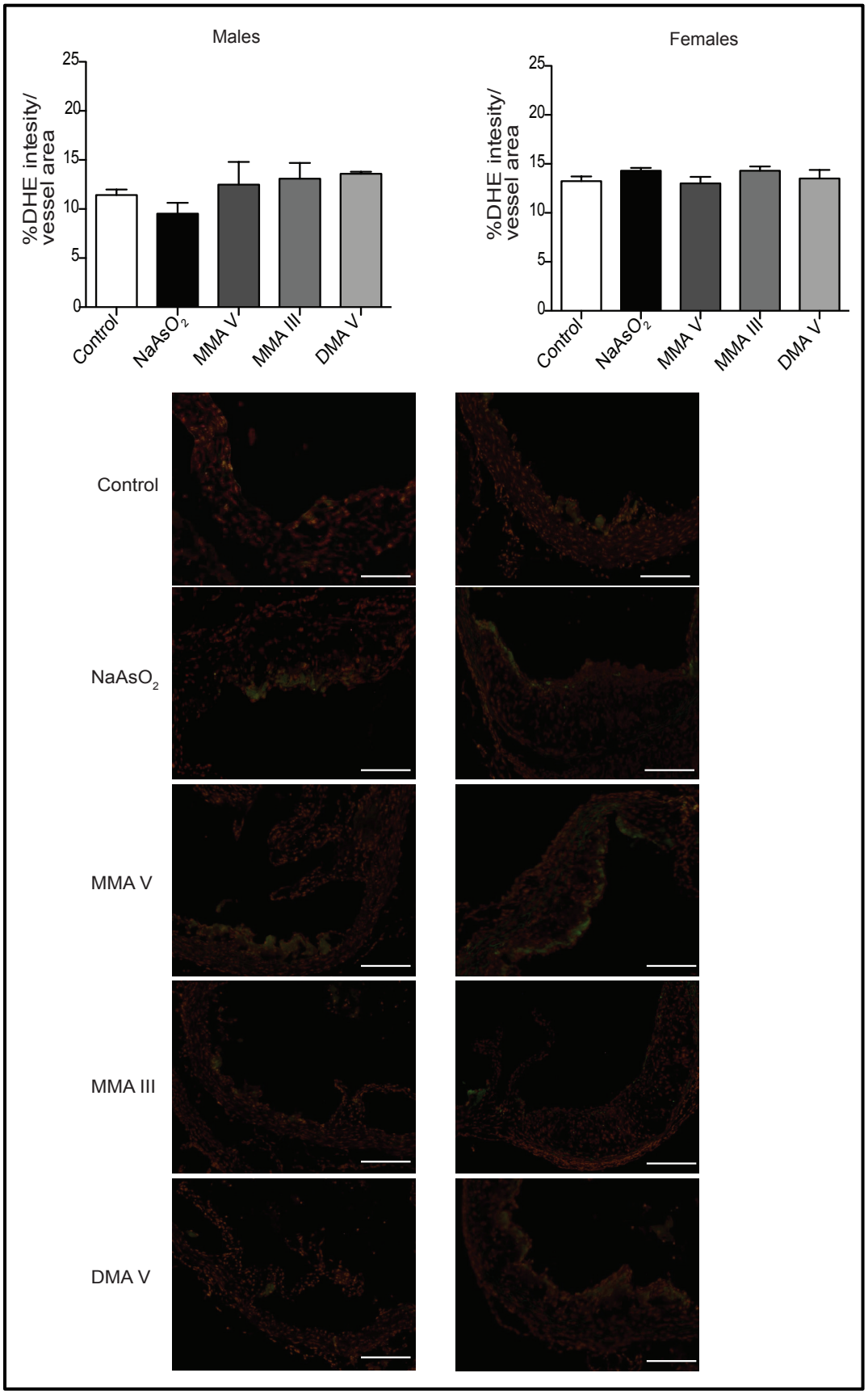
Males
%DHE intesity/ vessel area
25
20
15
10
5
0
Control
NaAsO2
MMA V
MMA III
DMA V
Females
%DHE intesity/ vessel area
25
20
15
10
5
0
Control
NaAsO2
MMA V
MMA III
DMA V
Control
NaAsO2
MMA V
MMA III
DMA V

Figure 3.3: Prenatal arsenic exposure does not increase ROS later in life.

ApoE$^{-/-}$ mice were exposed to 200 ppb arsenicals ($NaAsO_2$, MMA V, MMA III or DMA V) or maintained on tap water from conception to weaning. After weaning (4 weeks), male and female pups were kept on tap water for an additional 13 weeks. Frozen sections (at least 3 sections per animal; n>4 per group) of the aortic sinus were stained with 2 μM dihydroethidium to measure ROS levels. ROS levels were expressed as percentage of the total vessel area (A). Representative pictures are shown. Scale bar represents 200 μm. Values are expressed as mean ± SD.

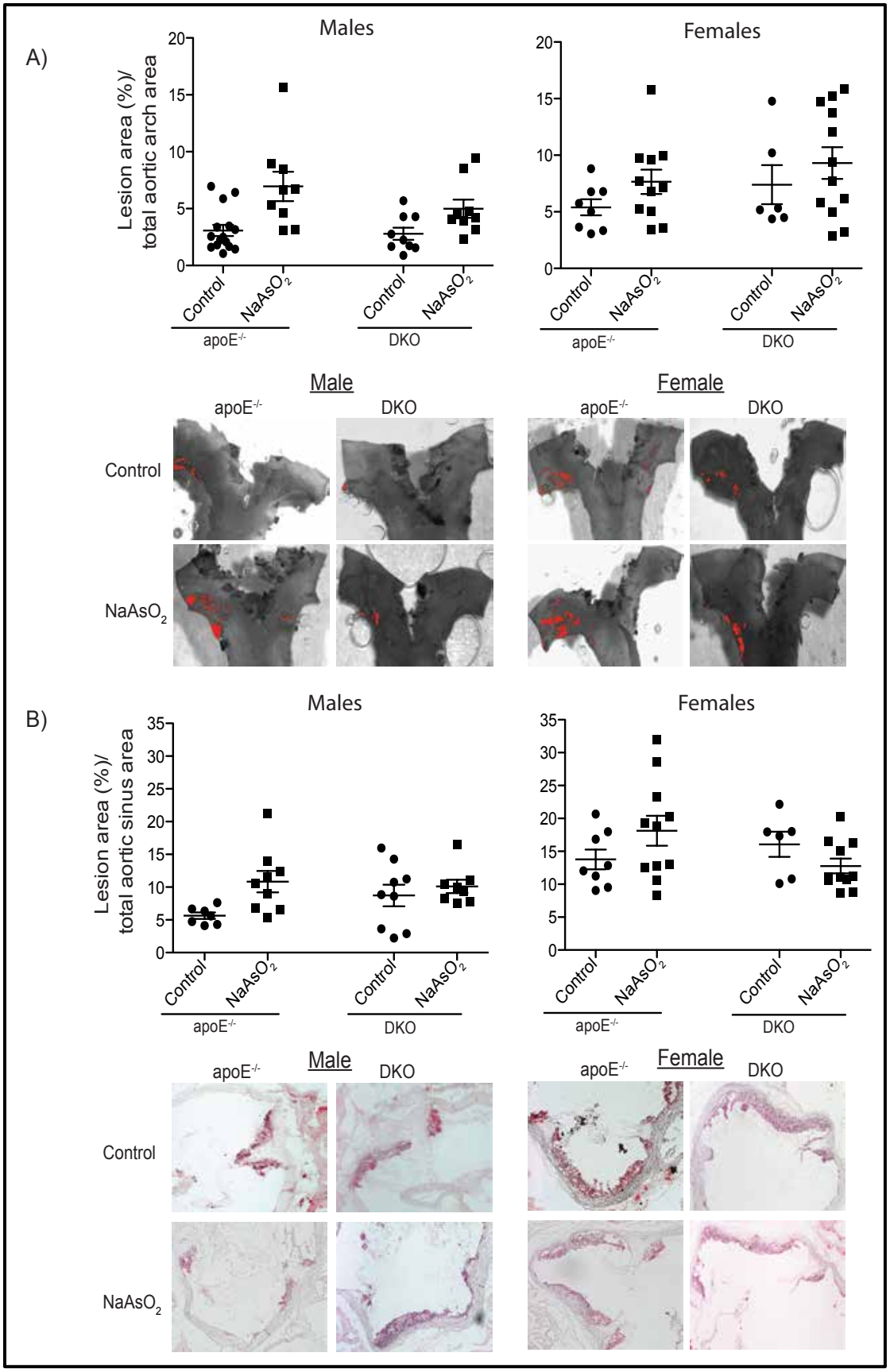
A)
Males
Females
Lesion area (%)/
total aortic arch area
20
15
10
5
0
Control
NaAsO2
apoE-/-
DKO
Male
Female
Control
NaAsO2
B)
Lesion area (%)/
total aortic sinus area
35
30
25
20
15
10
5
0

Figure 3.4: As3MT expression is required for arsenic-enhanced atherosclerosis later in life.

DKO mice were exposed to 200 ppb $NaAsO_2$ or maintained on tap water from conception to weaning. After weaning (4 weeks), animals were kept on tap water for an additional 13 weeks. Percent lesion area of the aortic arch (A) or aortic sinus (B) was evaluated via oil red O staining. Representative pictures are shown. Scale bar represents 100 μm. Values are expressed as mean ± SD.

Supplementary Table 3.1: Effect of arsenicals on plasma cholesterol, triglycerides, HDL, LDL, ALT and AST levels of apoE$^{-/-}$ male mice.

Groups	Cholesterol mmol/L	Triglycerides mmol/L	HDL mmol/L	LDL mmol/L	ALT U/L	AST U/L
Control	11.4 ± 2.2	1.2 ± 0.2	1.7 ± 0.2	9.1 ±2.0	56 ± 19	129 ± 32
NaAs	14.1 ± 3.8	1.4 ± 0.5	1.8 ± 0.3	11.6 ± 3.7	53 ± 10	104 ± 24
MMA V	15.5 ± 1.9	1.6 ± 0.5	2.0 ± 0.2	12.8 ± 1.7	56 ± 15	117 ± 23
MMA III	17.3 ± 3.3	1.9 ± 0.6	2.2 ± 0.3	14.3 ± 3.0	65 ± 20	111 ± 46
DMA V	15.2 ± 2.5	1.3 ± 0.3	2.0 ± 0.3	12.5 ± 2.2	61 ± 13	144 ± 46

Supplementary Table 3.2: Effect of arsenicals on plasma cholesterol, triglycerides, HDL, LDL, ALT and AST levels of apoE$^{-/-}$ female mice.

Groups	Cholesterol mmol/L	Triglycerides mmol/L	HDL mmol/L	LDL mmol/L	ALT U/L	AST U/L
Control	13.3 ± 0.4	1.5 ± 0.3	1.7 ± 0.1	10.8 ± 0.3	44 ± 5	104 ± 15
$NaAsO_2$	13.3 ± 4.1	1.4 ± 0.5	1.6 ± 0.2	10.9 ± 3.8	42 ± 7	111 ± 32
MMA V	18.2 ± 1.4	1.6 ± 0.5	1.7 ± 0.2	15.7 ± 1.7	38 ± 7	124 ± 44
MMA III	14.6 ± 6.2	1.6 ±0.6	1.7 ± 0.3	11.1 ± 4.5	43 ± 16	114 ± 20
DMA V	17.0 ±2.8	1.1 ± 0.2	1.6 ± 0.1	14.8 ± 3.0	58 ± 23	149 ± 89

Supplementary Table 3.3: Change in apoE$^{-/-}$ females compared to apoE$^{-/-}$ males in the plaque size and plaque components: macrophages, lipids, smooth muscle cells and collagen.

	Mean change (95% CI)	p value
Aortic Arch (%)	2.7 (1.0; 4.4)	**0.0020***
Aortic Sinus (%)	5.4 (2.9; 7.9)	**0.0000***
Macrophages (%)	1.3 (-04; 3.0)	0.1277
Lipids (%)	0.1 (-1.9; 2.1)	0.8908
SMC (%)	-0.9 (-2.7; 0.9)	0.3076
Collagen (%)	3.9 (1.2; 6.6)	**0.0054***

Supplementary Table 3.4: Change in DKO females from DKO males in the plaque size.

	Mean change (95% CI)	p value
Aortic Arch (%)	4.1 (1.6; 6.5)	**0.0021***
Aortic Sinus (%)	5.0 (2.0; 7.9)	**0.0019***

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CHAPTER 4

Arsenic exposure increases monocyte adhesion to the vascular endothelium, a pro-atherogenic mechanism

This work has been published previously, as indicate below:

Lemaire M*, Negro Silva LF*, Lemarié CA, Bolt AM, Flores Molina M, Krohn RM, Smiths, JE, Lehoux S, Mann, KK (2015). **Arsenic Exposure Increases Monocyte Adhesion to the Vascular Endothelium, a Pro-Atherogenic Mechanism**. PLoS ONE 10(9): e0136592. doi:10.1371/journal.pone.0136592

* Authors contributed equally to work

Although the present paper is a multi-authored paper, it has **not** been used in any other thesis.

4.1 Abstract.

Epidemiological studies have showed that arsenic exposure increases atherosclerosis, but the mechanisms underlying this relationship are unknown. Monocytes, macrophages and platelets play an important role in the initiation of atherosclerosis. Circulating monocytes and macrophages bind to the activated vascular endothelium and migrate into the sub-endothelium, where they become lipid-laden foam cells. This process can be facilitated by platelets, which favour monocyte recruitment to the lesion. Thus, we assessed the effects of low-to-moderate arsenic exposure on monocyte adhesion to endothelial cells, platelet activation and platelet-monocyte interactions. We observed that arsenic induces human monocyte adhesion to endothelial cells *in vitro*. These findings were confirmed *ex vivo* using a murine organ culture system at concentrations as low as 10 ppb. We found that both cell types need to be exposed to arsenic to maximize monocyte adhesion to the endothelium. This adhesion process is specific to monocyte/endothelium interactions. Hence, no effect of arsenic on platelet activation or platelet/leukocyte interaction was observed. We found that arsenic increases adhesion of mononuclear cells via increased CD29 binding to VCAM-1, an adhesion molecule found on activated endothelial cells. Similar results were observed *in vivo*, where arsenic-exposed mice exhibit increased VCAM-1 expression on endothelial cells and increased CD29 on circulating monocytes. Interestingly, expression of adhesion molecules and increased binding can be inhibited by antioxidants *in vitro* and *in vivo*. Together, these data suggest that arsenic might enhance atherosclerosis by increasing monocyte adhesion to endothelial cells, a process that is inhibited by antioxidants.

Keywords: arsenic, monocytes, CD29, VCAM-1, platelets, antioxidants

4.2 Introduction.

Arsenic is a tasteless and odorless environmental pollutant to which millions of people worldwide are exposed, mainly through consumption of contaminated food and water [1, 2]. The World Health Organization, the United States Environmental Protection Agency and Health Canada have set the maximum contamination level at 10 ppb in the municipal water [3], but well water in many areas can contain higher levels [2, 4]. For instance, over 10% of wells analyzed in Nova Scotia (Canada) have more than 500 ppb arsenic [5], and high endemic areas with levels up to 2.5 ppm are found in Bangladesh, the southwestern United States and Taiwan [2]. Growing epidemiologic evidence indicates that individuals exposed to arsenic, even at low concentrations, have an increased risk of developing cardiovascular diseases, including atherosclerosis [6-8]. Moreover, we and others have demonstrated that arsenic induces atheroma formation in apolipoprotein E-deficient ($apoE^{-/-}$) mouse models of atherosclerosis [9-11]. Interestingly, arsenic increased atherosclerosis without concomitant changes in circulating lipid profiles that could have contributed to the enhanced pathology [9]. Thus, the mechanisms by which arsenic exerts its effects remain uncertain.

Atherosclerosis is a multi-factorial disease resulting from a series of specific cellular and molecular events. Initiation of atherosclerosis involves endothelial cell activation by various stimuli, including cytokines, high levels of reactive oxygen species (ROS), and oxidized low density lipoprotein [12]. Arsenic has been described to participate in endothelial cell activation. In cultured endothelial cells, arsenic increases inflammatory molecule production [13], ROS [14, 15] and uptake of oxidized lipids [16], all of which have been linked to atherosclerosis. Once activated, the endothelial cells express adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1; CD106). VCAM-1 allows circulating mononuclear cells to firmly adhere to the endothelium and to transmigrate into the vascular wall [17, 18], and subsequent endothelial cell signaling generates low levels of ROS to support monocyte diapedesis [19]. In the arterial intima, monocytes differentiate to macrophages, engulf oxidized lipids, become lipid laden, and form foam cells, which contribute to the atherosclerotic core formation [20]. Therefore, monocyte binding to VCAM-1 is an essential step in atheroma formation. This process is facilitated by VCAM-1 binding to the *very late activation antigen-4* ligand (VLA-4; CD49d/CD29; hereafter called CD29)

expressed by leukocytes. VLA-4 is an integrin receptor of the β1 family that selectively binds to VCAM-1 [21].

Although arsenic-mediated pro-atherogenic effects on macrophages have been described, including impaired cytokine secretion and immune responses [22, 23], little is known about monocytes as cellular targets for arsenic. Similarly, neutrophils are found along the aortic vascular wall during atherosclerosis in apoE$^{-/-}$ mouse model of atherosclerosis [24], and activated platelets are reported to increase monocyte adhesion to the vascular lesions and enhance plaque formation [25], but the effects of arsenic on these cells remains unknown.

Here, we hypothesized that arsenic may affect early events in atherogenesis. Thus, we assessed the effects of a low-to-moderate arsenic exposure on monocyte, neutrophil, platelet, and endothelial cell interactions as potential pro-atherogenic mechanisms.

4.3 Material and Methods.

4.3.1 Chemicals.

We utilized two trivalent inorganic arsenic compounds for these studies. To compare with established literature, arsenic trioxide (FW 197.84 g/L; As_2O_3) (Sigma-Aldrich, Oakville, Ontario, Canada) was used for all the *in vitro* assays. When dissolved in NaOH, arsenic trioxide will form arsenite (Balanced equation: $As_2O_3 + 2\ NaOH = H_2O + 2\ NaAsO_2$) [26]. Therefore, it was dissolved in 0.1 N NaOH and subsequently diluted in sterile phosphate buffer saline solution (PBS) prior addition to the cells. However, because of its greater dissolution index in water, *m*-sodium arsenite (FW 129.91 g/L; $NaAsO_2$) (Sigma-Aldrich) was used as a source of arsenic for the *in vivo* exposures [26]. Thus, we used the *ppb* nomenclature to compare the concentration of the arsenic molecules in solution from these two sources of arsenic. Antioxidants N-acetylcysteine [27] is from Sigma-Aldrich.

4.3.2 Cell culture.

Human monocytic U937 cells (ATCC CRL-1593.2; Manassas, Virginia, USA) and human peripheral blood primary monocytes were cultured in RPMI-1640 medium (Invitrogen Inc., Ontario, Canada). Human peripheral blood primary mononuclear cells (PBMC) were differentiated into macrophages with macrophage colony stimulating factor (M-CSF; 50 ng/ml; PeproTech, NJ, USA) for 12 days. Human cells were obtained after participants provided their written informed consent using a protocol approved by the Research Ethics Review Board (REB) of the Jewish General Hospital. Murine bone marrow primary monocytes were cultured in RPMI-1640 medium containing 5% β-mercaptoethanol (Sigma-Aldrich). Human umbilical vein/vascular endothelium (HUVEC) cells were kindly provided by Dr. Mark Blostein (Lady Davis Institute for Medical Research, Montréal, Qc, Canada), which acquired those cells from ATCC (CRL-1730) and sustained in F-12K medium (ATCC) containing 0.1 mg/ml heparin (Sigma-Aldrich), 0.03 mg/ml endothelial cell growth supplement (AbD Serotec, Raleigh, NC, USA) on 0.1% gelatin-coated plates. All cells were cultured in medium containing 10% fetal bovine serum (FBS; Wisent, St-Bruno, Qc, Canada) and penicillin/streptomycin (Wisent) at 37°C with 5% CO_2.

4.3.3 Animals.

Wild-type C57BL/6 and B6.129P2-*apoe*^{tm1Unc}/J (apoE$^{-/-}$) male mice were obtained from Jackson laboratory (Bar Harbor, Maine, USA). The McGill Animal Care Committee permitted the experimental protocol and animals were handled in accordance with institutional guidelines, which followed the Canadian Council of Animal Care. The McGill Animal Care Committee is IACUC approved. For long-term arsenic exposure experiments, apoE$^{-/-}$ male mice (3 week old, n = 5 animals per group) were grouped with cage companionsand fed *ad libitum* with normal rodent chow (2018; Harlan Laboratories Inc., WI, USA)or deficient (0.009 mg/kg) or high (0.3 mg/kg) selenium-containing lentil diet (Krohn *et al*, *in revision*) at the animal facility. The normal rodent chow contains 0.2 mg/kg selenium (2018; Harlan Laboratories). Low levels of arsenic were detected both in the tap water (0.75 ppb) and in the normal rodent chow (1.90 ppb) ± S.D. of 5%, with detection limit of 0.65 ppb [9]. Starting at 5 weeks of age, mice were either maintained on tap water or on tap water-containing 200 ppb arsenic (0.35 mg/L $NaAsO_2$) for 8 or 13 weeks, as described previously [9]. We decided to feed the animals with selenium-containing food instead of adding the selenium in the water. This prevents formation of arsenic-selenium complexes in the water. As we have previously described [9] no obvious toxicities were observed in mice given arsenic at any time during the experiment.

4.3.4 Plasma analyses.

Blood (0.6 ml) was collected using EDTA-coated tubes (Sarstedt, Germany) by cardiac puncture and plasma was recuperated. Circulating levels of the chemokine CCL5 (RANTES) and CCL2 (MCP-1) were measured using an immunoassay kit (multiplex bead-based) on a Bio-Plex 200 (Bio-Rad Laboratories, ON, Canada), as previously described [9]. Each sample ($n \geq 4$ animals per group) was analyzed in duplicate.

4.3.5 Isolation of primary human and murine cells.

In order to obtain human primary monocytes, blood samples (50-100 ml) were collected from healthy normal donors in tubes coated with sodium heparin (BD Vacutainer). These cells were obtained after participants provided their written informed consent using a protocol approved by the REB of the

Jewish General Hospital. The REB also approved the procedures. Samples were centrifuged for 10 min at 1200 rpm to separate the plasma from the cells. Cells were diluted in HBSS medium (Wisent), slowly layered onto a Ficoll solution (GE Healthcare Life Sciences, Baie d'Urfé, Qc, Canada) and centrifuged at 2200 rpm for 30 min. The medium layer containing mononuclear cells was collected and diluted in HBSS medium. Cells were centrifuged again for 10 min at 1200 rpm and the pellets were collected and washed in HBSS. Peripheral blood mononuclear cells were seeded in RPMI-1640 + 10% FBS and allowed to adhere to plastic for 1.5 h in order to enrich the monocytic population [28]. The supernatant containing non-adherent cells was removed and fresh RPMI-1640 + 10% FBS was added to the attached monocytes.

To isolate wild-type murine monocytes from bone marrow, tibiae from C57BL/6 mice were flushed with RPMI-1640 medium. Cells were homogenized with 18G needle, centrifuged and suspended in RPMI-1640 + 10% FBS in single cell suspension prior to performing monocyte-enriching adherence step.

4.3.6 Platelets preparation, measurement of platelets activation and assessment of platelets aggregates with neutrophil or monocytes.

To study the effects of arsenic exposure on platelet activation in context of atherosclerosis, we collected platelets from C57Bl/6 and apoE$^{-/-}$ mice. Blood samples were taken from the saphenous vein using EDTA-coated tubes (Sarstedt, Germany). Blood was diluted 1:1 with flow cytometry buffer, consisting of PBS supplemented with 5% FBS and 0.01M sodium azide, and centrifuged at 60 g for 10 min to recuperate the platelet rich plasma (PRP). PRP was further centrifuged at 240 g for 10 min, and the pellet was suspended in flow cytometry buffer.

In order to evaluate platelet activation, 5 x 10^6 platelets were seeded on fibrinogen-coated (30 ug/mL) coverslips in wash buffer [150 mM NaCl, 20 mM PIPES (Sigma-Aldrich), pH 6.5]. Platelets were then exposed for 5 min to arsenic or human α-thrombin, a platelet activator (1U, Haematologic Technologies Inc, Essex Junction, Vermont, USA). The thrombin was aliquoted in sterile PBS, kept at -20°C, and used fresh for every experiment. Once rinsed, PRP was exposed to 0.1% Triton X-100 (Amresco, Solon, Ohio, USA) at room temperature in the dark for 1 h. Triton was removed, and PRP was blocked with BSA/PBS 0.1% Tween 20 (Bio-Rad Laboratories) for 1 h. Blocking media was removed, and

PRP was stained with fluorescent dye (Phalloidin; Alexa Fluor; Molecular Probes; Life Technologies, Burlington, ON, Canada) for 2 h in the dark. Images for platelet spreading were acquired using *Infinity Capture* software and camera (Lumenera, Canada). Assessment of surface platelet activation biomarker was also performed. 5 x 10^6 platelets were centrifuged at 1000 rpm for 5 min. Arsenic and thrombin were added directly to the pellet, for 5 min. After incubation, platelets were washed with flow cytometry buffer (0.5 ml) prior to assess cellular surface biomarkers of activation, as described below.

In addition to platelet activation assays, platelet/monocyte and platelet/ neutrophil aggregates were detected *in vivo* in order to evaluate the effects of arsenic exposure on their formation. $CD14^+/CD41^+$ platelet/monocyte and $Ly6G^+/CD41^+$ platelet/neutrophil aggregates were monitored at 14, 21 and 28, or 21, 28 and 35 days of arsenic exposure, respectively. Blood was collected as for platelet activation assays, and surface markers detected as described below. One mouse received lipopolysaccharide (LPS; 1 mg/kg; Sigma-Aldrich) for 18 h as a positive control.

4.3.7 Cellular surface antigen assessment.

Surface antigens were detected by direct immunofluorescence using flow cytometry (Beckman Coulter, Mississauga, Ontario, Canada and LSRFortessa Cell Analyzer (BD Biosciences, San Jose, California, USA). Briefly, control cells and cells exposed to arsenic were washed twice with PBS supplemented with 5% FBS (Wisent) and 0.01 M sodium azide (flow cytometry buffer). The cells were then exposed to labeled anti-CD29 (eBioscience anti-murine; Pharmingen anti-human: HUTS-21 clone), P-selectin (BD Pharmingen), CD14 (eBioscience), CD41 (BD Pharmingen), Ly6G (eBioscience) or their specific isotype control antibody and incubated for 45 min on ice in the dark. Cells were then washed twice with flow cytometry buffer, fixed in 2% paraformaldehyde and analyzed by flow cytometry. The gates for positive-staining cells were determined by comparison with cells stained with the isotype-matched control antibodies. The FCS express and FlowJo softwares (denovosoftware, CA, USA; flowJo LLC, Ashland, Oregon, USA) were used to analyze the data.

4.3.8 Cell adhesion assay.

Cell culture plates were coated overnight with recombinant mouse VCAM-1/Fc chimera (2 μg/mL; R&D Systems; MN, USA) at 4°C, rinsed with PBS and saturated with 2% BSA for 1 h. Human and murine primary circulating monocytes or U937 cells, were incubated with CellTracker orange CMTMR fluorescent dye (0.25 μl/ml; Molecular Probes; Life Technologies) in RPMI-1640 for 30 min, centrifuged and resuspended in RPMI-1640 + 10% FBS culture medium. Fluorescently-labelled cells (1000 cells/well) were incubated with the VCAM-1/Fc chimera-coated plates for 30 min with or without CD29 blocking antibody (0.5 μg; BD Pharmingen, clone 9EG7). Non-adherent cells were washed twice with PBS, and the adherent fluorescent cells were counted under a fluorescence microscope.

To assess monocyte adhesion to endothelial cells, HUVEC cells were placed on 0.1% gelatin-coated cover slide. At 80% confluence, cells were exposed to arsenic for 72 h. Arsenic-exposed, fluorescently-labelled U937 cells (1000 cells/ml) were seeded over HUVEC and allowed to adhere for 1 h. The non-adherent cells were washed away, and the co-culture was stained with DAPI 1:2 into mounting media (Vectashield h-2000, Vector Laboratories: Immu-Mount, Thermo Scientific, respectively) and the adherent fluorescent cells were counted under a fluorescence microscope and expressed relative to the total number of cells per field.

4.3.9 Organ culture.

Bone marrow and carotids were isolated from C57BL/6 12-week-old male mice. The carotids were connected to a perfusion circuit consisting of a 3-port reservoir, a pump and a pressure chamber, as previously described [29, 30]. The arteries were immersed in the chamber in DMEM (Invitrogen Inc) + 5% FBS culture medium, and the circulation initiated overnight with medium with and without arsenic. In parallel, the isolated bone marrow cells were also cultured overnight in RPMI-1640 with 10% FBS with and without arsenic. These cells were then fluorescently-labelled (CellTracker orange CMTMR; Life technologies) and injected in the carotids [31]. They were allowed to adhere to the endothelial cells of the vasculature wall for 30 min before being washed. In some experiments, CD29 blocking antibody (0.5 μg) was added for 30 min prior to the bone marrow cell injection in the system. Total adherent cells were

counted under a fluorescent microscope and results are expressed relative to surface area (number of cells / [$2\pi rh + 2(\pi r^2)$] * 10^6; r = radius, h= length of the vessel).

4.3.10 Immunohistochemical analysis.

ApoE$^{-/-}$ murine carotids arteries were removed (n=4), rinsed, fixed in 4% paraformaldehyde and incubated overnight in a 30% sucrose solution, as previously described [9]. Carotids were then frozen in Tissue Tek OCT (Sakura, CA) compound, and serial cryosections of 6-µm thickness were sliced. Carotids were stained with primary antibodies against VCAM-1 (sc-8304; Santa Cruz Biotechnology, Dallas, TX, USA). Briefly, four to six sections/animal were incubated with primary anti-VCAM-1 antibodies (1:200) at room temperature. Biotinylated-secondary antibody (1:500; BA-1000; Vector Laboratories, Burlingame, California, USA) was incubated at room temperature for 30 minutes, then slides were processed using the peroxidase VECTASTAIN ABC kit (Vector Laboratories) and developed with ImmPACT^{TM} DAB peroxidase substrate (Vector Laboratories). Sections were counterstained with 2% Harris modified hematoxylin (Thermo Fisher Scientific, Waltham, Maine, USA) and mounted in Permount (Thermo Fisher Scientific). Images were acquired using *Infinity Capture* software and camera (Lumenera). VCAM-1 is expressed as the number of positive cells relative to the carotid luminal perimeter using ImageJ software (NIH).

4.3.11 Detection of superoxide.

U937 cells were pre-treated with the antioxidant NAC (1 mM) for 1 h. Media was changed, and the cells (1 x 10^6 cells) were exposed to arsenic or vehicle control for 3 h. Cells were then stained with 2 µM hydroethidine (HEt; Molecular Probes) in PBS supplemented with 1% FBS for 30 min at 37°C. Cells were washed with warm PBS, and analyzed by flow cytometry (Beckman Coulter).

Carotids from ApoE$^{-/-}$ mice fed either deficient or high-selenium food pellets were removed, fixed, frozen and sliced as previously described [9]. Het (0.1 mM) diluted in PBS was added directly on the frozen tissue, and pictures were acquired using *Infinity Capture* software and camera (Lumenera, Canada). Positive staining was expressed as percentage of vascular wall area from at least 3 sections per animal (n=4 animals) using Image J software (NIH).

4.3.12 Statistical considerations.

For statistical analysis, the one-way ANOVA was performed and the p value was evaluated with a Tukey's post hoc test using the GraphPad Instat software (San Diego, CA, USA). A p value < 0.05 indicates statistical significance. The data correspond to the mean values ± S.D.

4.4 Results.

4.4.1 Arsenic induces monocyte adhesion to endothelial cells, with maximal binding achieved following exposure of both cell types.

The interaction between monocytes and vascular endothelium is one of the initial events in atherosclerotic plaque formation. We hypothesized that arsenic increased this interaction as part of its pro-atherogenic mechanisms. In order to assess the effect of arsenic on monocyte adhesion to endothelial cells, we studied the adhesion *in vitro* and *ex vivo*. First, we assessed binding of U937 monocytic cells to HUVECs, where either cell type or both were exposed to arsenic for 72 hours (10 or 200 ppb). While arsenic exposure of HUVECs alone did not increase adhesion of U937 cells, U937 exposed to 200 ppb arsenic adhered significantly more to untreated HUVECs (Figure 4.1A and 4.1B). Interestingly, when both cell types were exposed to arsenic, there was significantly more binding. This was most marked for the lowest concentration of arsenic. While 10 ppb arsenic exposure of either cell type alone did not increase adhesion, it significantly enhanced binding when both cell types were treated (Figure 4.1A).

Next, we extrapolated these findings to an *ex vivo* model using murine carotid arteries and bone marrow cells [31]. This model allowed us to determine interactions between primary mononuclear cells and vascular endothelium on its native basal lamina. This is particularly important considering data that endothelial cell inflammatory signalling can differ depending on the extracellular matrix used in culture [32]. The vessel, the total bone marrow cells, or both were exposed to arsenic. The fluorescently-labelled, bone marrow cells were subsequently injected into the carotid and allowed to adhere to the vascular endothelium for 30 min. We found that, as with our *in vitro* cultures, exposure of both the endothelium and the bone marrow cells leads to maximal binding (Figure 4.1C). Furthermore, *ex vivo* adhesion is dose-dependent and is achieved at arsenic concentrations, as low as 10 ppb (Figure 4.1D) when both components are exposed to arsenic.

4.4.2 Arsenic does not enhance platelet activation, platelet/monocyte or platelet/ neutrophil interactions.

In addition to endothelial cell/monocyte interactions, adhesion of monocytes and neutrophils to circulating platelets contributes to plaque formation [24, 25]. Platelets, once activated, induce cytokine secretion from the endothelium to recruit leukocytes to the vasculature. Hence, we assessed the effects of low-to-moderate arsenic exposure on platelet activation, and on platelet/monocyte and platelet/neutrophil aggregation. First, we determined whether arsenic could activate platelets from C57BL/6 or apoE$^{-/-}$ mice, as has been shown in wild-type rats [33]. We were particularly interested in apoE$^{-/-}$ mice, because this hyperlipidemic strain was used in our *in vivo* model of arsenic enhanced atherosclerosis [9]. We measured platelet activation either by spreading morphology or P-selectin expression, a surface marker of platelet activation (reviewed in [34]). In contrast to reported data [33], our results showed that arsenic alone did not activate murine C57Bl/6 or apoE$^{-/-}$ platelets in either assay (Figures 4.2A and 4.2B), whereas thrombin, a known platelet agonist, induced platelet spreading and P-selectin expression. However, arsenic did not alter thrombin-induced platelet activation. Additionally, we assessed the circulating levels of CCL5, a cytokine secreted from activated platelets that promotes early recruitment of monocytes and neutrophils to the endothelium [35]. CCL5 levels were assessed in plasma from arsenic-exposed apoE$^{-/-}$ mice after 8 and 13 weeks, time points where significant arsenic-enhanced atherosclerosis is observed [9]. However, CCL5 levels were unchanged by arsenic at the time of the early 8-week lesion and the well-established 13-week lesion (Figure 4.2C).

Monocyte- and neutrophil-platelet aggregates are recruited to the activated vascular endothelium early in the atherosclerotic process. Thus, we evaluated the circulating platelet/monocyte and platelet/neutrophil aggregates in the whole blood of apoE$^{-/-}$ mice treated over 28 days with 200 ppb arsenic, a concentration known to cause a significant increase in plaque formation [9]. No significant changes were observed over this period in either monocyte or neutrophil aggregation with platelets. However, the LPS-exposed mice, used as positive control, had a significantly higher platelet/monocyte aggregate formation (Figure 4.2D). Together, these data indicate that arsenic specifically enhances the interactions between monocytic cells and the endothelium, but not the interactions of leukocytes with platelets.

Circulating levels of CCL2 (MCP-1) can enhance monocyte/endothelial cell adhesion [36]. Thus, we measured CCL2 in the plasma of apoE$^{-/-}$ mice given 200 ppb arsenic orally for either 8 or 13 weeks. No significant changes in circulating levels of CCL2 in mice exposed to arsenic as compared to tap water controls (Supplementary Figure 4.1). These levels were also not elevated compared to CCL2 levels observed in wild-type C57BL/6 mice. This suggests that CCL2 levels are not increased by arsenic in this model of atherosclerosis.

4.4.3 Arsenic induces mononuclear cell adhesion to VCAM-1 via CD29.

By binding to VCAM-1, VLA-4 mediates the attachment of circulating cells to the activated vascular endothelium, favoring monocyte migration into the subendothelial space. VLA-4 is composed of CD49d (α4) and CD29 (β1) integrins. Interaction of monocyte VLA-4 with VCAM-1 occurs via inducible interactions between CD49d and CD29, producing changes in affinity (structural) or avidity (number) [37]. We tested whether arsenic exposure of monocytes could increase binding to immobilized VCAM-1. We observed that both human U937 monocytic cells and primary human monocytes adhered more to the VCAM-1/Fc chimera when exposed to arsenic for 72 h, as compared to the non-exposed control (Figure 4.3A). Furthermore, the presence of arsenic during the monocyte differentiation into macrophages also resulted in greater adhesion to VCAM-1 (Figure 4.3A). CD29 is constitutively expressed on monocytes [38], but upon activation, adopts an active conformation and its CD49a binding sites (the HUTS epitopes) become available for the antibody. Active-CD29 expression was slightly, but significantly, increased on human primary macrophages, but not monocytes, following arsenic exposure (Figure 4.3B; *: $p < 0.05$). We confirmed that CD29 was responsible for arsenic-increased binding to VCAM-1 by the addition of an anti-CD29 blocking antibody. Our results show a significant inhibition of U937 cell adhesion to VCAM-1 when blocking CD29 (Figure 4.3C; **: $p < 0.01$). We observed that the CD29 blocking antibody also prevented arsenic-induced binding of leukocytes to vascular endothelium in our *ex vivo* organ culture model (Figure 4.3D), supporting an important role for the VCAM-1/CD29 interaction in mediating the initial monocyte/endothelial cell interaction.

4.4.4 Antioxidants can block binding of monocytes to VCAM-1 *in vitro* and *in vivo.*

Arsenic is a potent inducer of reactive oxygen species (ROS) and many of the effects of arsenic have been attributed to ROS production [14, 15]. In addition, VCAM-1 expression and signaling is known to be regulated through ROS [19, 39]. Thus, we studied the role of arsenic-induced ROS in our models using NAC [40]. NAC increases the intracellular glutathione (GSH) pool, which can itself act as an antioxidant or can bind arsenic to increase its export [40]. First, we demonstrated that arsenic-increased ROS can be prevented *in vitro* by pre-treating U937 cells with NAC (Figure 4.4A). Second, we tested whether the increased binding of U937 cells to VCAM-1/Fc was dependent upon ROS. U937 cells were pre-treated for 1 hour with NAC, followed by 3 hours with arsenic before binding to VCAM-1/Fc was assessed. NAC significantly inhibited U937 cell binding to VCAM-1 (Figure 4.4B; *: $p < 0.05$), indicating that ROS mediate arsenic-increased adhesion of mononuclear cells to VCAM-1.

To extend our *in vitro* findings, we utilized our *in vivo* model of arsenic-enhanced atherosclerosis to determine whether arsenic exposure correlated with increased adhesion molecule expression. Thus, we compared the expression of VCAM-1 on the endothelium of carotid arteries from tap water and arsenic-exposed, apoE$^{-/-}$ mice. Indeed, endothelium from arsenic-exposed mice expressed significantly more VCAM-1 than the control group (Figure 4.5A). In addition, circulating monocytes of apoE$^{-/-}$ mice exposed to arsenic expressed significantly more active-CD29 than their control counterparts (figure 5B).

Finally, to evaluate the contribution of arsenic-induced ROS adhesion molecule expression and function *in vivo*, we co-exposed apoE$^{-/-}$ mice to arsenic along with the antioxidant selenium. We chose to utilize this antioxidant, because we could incorporate variable levels in the animal chow (high and low selenium diet), in order to avoid interactions between the antioxidant and arsenic in the drinking water. We first confirmed that high levels of selenium prevented arsenic-induced ROS *in situ* in the carotids when compared to arsenic-exposed mice on a low selenium diet or unexposed mice (Figure 4.5C; *: $p < 0.05$). Arsenic-enhanced VCAM-1 staining was prevented when the animals were fed a diet-containing a high level of the antioxidant selenium (Figure 4.5D). Interestingly, active-CD29 levels on circulating monocytes were increased 2-fold in arsenic-exposed mice on low selenium diet, but this was abrogated in mice receiving a high selenium diet (Figure 4,5E). Elevated levels of active-CD29 correspondingly resulted in increased adhesion of circulating monocytes, when monocytes were removed from exposed

mice and cultured on VCAM-1/Fc (Figure 4.5F). Together, these data suggest that arsenic-induced ROS likely plays a role in the increased endothelial VCAM-1 and mononuclear cell active-CD29 expression, which favors pro-atherogenic adhesion.

4.5 Discussion.

Despite compelling evidence that links environmental arsenic exposure to an increased risk of atherosclerosis [6-8], the mechanisms by which arsenic enhances atherosclerosis remain to be fully established. We hypothesized that it may increase pro-atherogenic cellular interactions important in the initial phases of the pathology. In this study, we demonstrate that arsenic exposure specifically enhances mononuclear-endothelium interactions *in vitro* and *ex vivo*. Arsenic increases VCAM-1 and CD29 adhesion molecules to increase monocyte binding, which can be prevented *in vitro* and *in vivo* by antioxidants. In fact, we observed that arsenic exposure specifically enhanced monocyte attachment to endothelial cells (Figure 4.1), but failed to induce platelet activation and aggregation with leukocytes (figure 2). In our apoE$^{-/-}$ mouse model of atherosclerosis, arsenic increased vascular expression of VCAM-1 and monocyte expression of active CD29 (Figure 4.5). Interestingly, arsenic-induced VCAM-1 expression and monocyte adhesion could be prevented by co-exposure with the dietary antioxidant selenium (Figure 4.5). Thus, we believe that in part, arsenic-induced pro-atherogenic mechanisms are linked to enhanced circulating monocyte adhesion to the endothelium through generation of ROS.

Several of the mechanisms implicated in arsenic-enhanced atherosclerosis are associated with endothelial activation, such as production of inflammatory molecules [11, 23] and ROS [14, 15]. The first adhesion molecule expressed upon endothelial cell activation is VCAM-1, which is virtually absent on the vasculature prior to activation [41]. VCAM-1 expression is uniquely upregulated upon atherosclerotic stimuli [42], and exacerbation of cellular recruitment to VCAM-1 contributes to atherosclerosis [43]. ICAM-1 (intercellular adhesion molecule-1) is also expressed upon endothelial activation [41, 42, 44] but, VCAM-1 is the major player in the establishment of the nascent lesion [45]. Hyperlipidemic Ldlr$^{-/-}$ mice lacking the VCAM-1 extracellular domain displayed decreased plaque formation compared to Ldlr$^{-/-}$ mice with wild-type VCAM-1, while deletion of ICAM-1 did not alter the early plaque formation [45]. Both human and murine atherosclerotic vascular lesions express VCAM-1, which correlates with the extent of exposure to the pro-atherogenic stimuli [46]. Thus, we focused our investigations on VCAM-1, and found that atherogenic concentrations of arsenic [9, 47] induced vascular endothelial VCAM-1 expression at lesion-prone sites in apoE$^{-/-}$ mice model of atherosclerosis (Figure 4.5A). Interestingly, this supports the positive association observed between arsenic exposure and increased human soluble VCAM-1

concentration in the plasma [48, 49], which is known to increase with endothelium activation in atherosclerosis [50].

Our *in vitro* and *in vivo* data support the hypothesis that increased VCAM-1/integrin adhesion is dependent upon ROS. Antioxidants inhibited arsenic-induced monocyte binding to VCAM-1 *in vitro* (Figure 4.4). A high selenium diet decreased VCAM-1 expression on vascular endothelial cells and decreased active-CD29 expression on monocytes *in vivo*, associated with decreased monocyte binding capacity to VCAM-1 (figure 5). Selenium is an essential micronutrient, and acts as an antioxidant through its action as a co-factor for enzymes, such as GSH peroxidase and thioredoxin reductase [51]. It also binds arsenic to form the seleno-bis(S-glutathionyl) arsinium ion, which enhances excretion through the hepatobiliary system [52]. Thus, selenium co-exposure will increase not only the total antioxidant capacity, but also the clearance of arsenic, which both should prevent ROS damage.

The role of ROS in mediating increased adhesion may be multi-fold. ROS may play a role in VCAM-1 expression via NFκB activation [53]. Interestingly, arsenic induces NFκB [54], which might be responsible for the observed arsenic-increased VCAM-1 expression in our apoE$^{-/-}$ mice model (Figure 4.5). The binding of VCAM-1 to the VLA-4 integrin rapidly activates the production of low concentrations of H_2O_2 in the endothelial cell [19] that signals to activate matrix metalloproteinases-2 and -9 (MMP-2/9) [39]. MMPs degrade extracellular matrix and cleave endothelial cell junctions to allow monocyte diapedesis [51, 55]. We previously observed that arsenic exposure slightly, but not significantly, induces MMP activity within the apoE$^{-/-}$ atheromas [47]. Thus, arsenic-induced ROS could promote the monocyte transmigration through the endothelial cell layer by enhancing VCAM-1 expression even in the absence of a supplementary effect on MMPs.

We demonstrated here that arsenic exposure enhances monocyte interaction of VLA-4 with endothelial adhesion molecule VCAM-1 (Figure 4.1). Increased binding might be the critical step for arsenic-induced plaque formation *in vivo*, because circulating monocyte firm adhesion to the endothelium is required for atherosclerosis formation [43]. Inhibition of VLA-4 with blocking antibodies has been shown to prevent monocyte adhesion in apoE$^{-/-}$ fed high fat diet [56, 57]. We confirmed that utilization of blocking antibody targeting CD29 specifically prevents arsenic-induced monocytes adhesion to VCAM-1, suggesting the biological importance of this integrin in arsenic-induced atherosclerosis. Although present

on lymphocytes, VLA-4 integrin is responsible for the specific monocytic binding to VCAM-1 [42]. Interestingly, arsenic-exposed apoE$^{-/-}$ mice displayed increased monocytic active-CD29 expression, which correlates with increased vascular VCAM-1 expression (Figure 4.5). This might suggest that arsenic is able to trigger activated VLA-4 conformation itself. This is even more likely when we consider that in order to have maximal binding *in vitro* or *ex vivo*, both mononuclear and endothelial cells need to be exposed to arsenic (Figure 4.1). While CD29 is constitutively expressed in monocytes [38], the regulation of its active form is proposed to arise from either β2 chain integrin engagement [58], via modification in CD49d α4 transcription level [59] or through ROS-dependent mechanisms [60]. We previously assessed CD49d α4 expression and saw no change (unpublished data), thus we focused on arsenic-mediated ROS effects. Interestingly, the antioxidant NAC was efficient in preventing ionizing radiation-induced CD29 β1 membrane expression in murine RAW.267 macrophages [60]. The authors proposed that it might explain atherosclerosis formation observed in cancer patients receiving ionizing radiation. Further investigation is needed to understand arsenic-induced regulation of CD29 integrin, but interestingly, we observed that *in vivo* arsenic-enhanced CD29 expression is prevented when apoE$^{-/-}$ mice are co-exposed to selenium, which suggests that arsenic may control CD29 regulation through ROS production (Figure 4.5).

Although we found that arsenic enhanced monocyte/endothelial cell adhesion, we found no evidence, from either wild-type or atherogenic mice, of increased platelet activation or platelet/leukocyte aggregates, all of which are involved in plaque initiation [35, 61, 62]. This contrasts with previous reports that arsenic induces platelet activation and aggregation *in vit*ro and *in vivo,* resulting in enhanced arterial thrombosis [33]. However, we exposed to much lower concentrations of arsenic [up to a maximum of 200 ppb sodium arsenite (2.6 μM arsenic) for 5 min], whereas previous reports indicate that platelets require much higher concentrations of arsenic (above 10 μM) to reach activation at significantly longer exposure periods. Furthermore, we did not expose platelets to an inhibitor of platelet aggregation prior to arsenic exposure, which also might explain part of the discrepancies. To confirm our data *in vivo*, we exposed apoE$^{-/-}$ mice to pro-atherogenic arsenic concentration, and assessed circulating levels of CCL5, a major cytokine secreted upon platelet activation [63] that contributes to mononuclear cell accumulation at the lesion site in this model [64]. However, arsenic exposure did not alter CCL5 plasma concentrations

(Figure 4.2). Furthermore, we found no evidence of increased circulating platelet/leukocyte aggregates over one month exposure to arsenic (Figure 4.2), suggesting limited participation of platelets in arsenic-increased atherosclerosis. This highlights the specificity of increased monocyte/endothelial cell adhesion following arsenic exposure, rather than implicating a general increase in intercellular interactions.

Together, our data suggest that arsenic promotes monocyte adhesion to the endothelial cells, providing a possible mechanism for the effect of arsenic on atherosclerosis. Furthermore, our studies indicate that these early events in atherosclerosis induced by arsenic can be prevented by antioxidants. Thus, prevention of atherosclerosis may be possible in high risk populations exposed to arsenic.

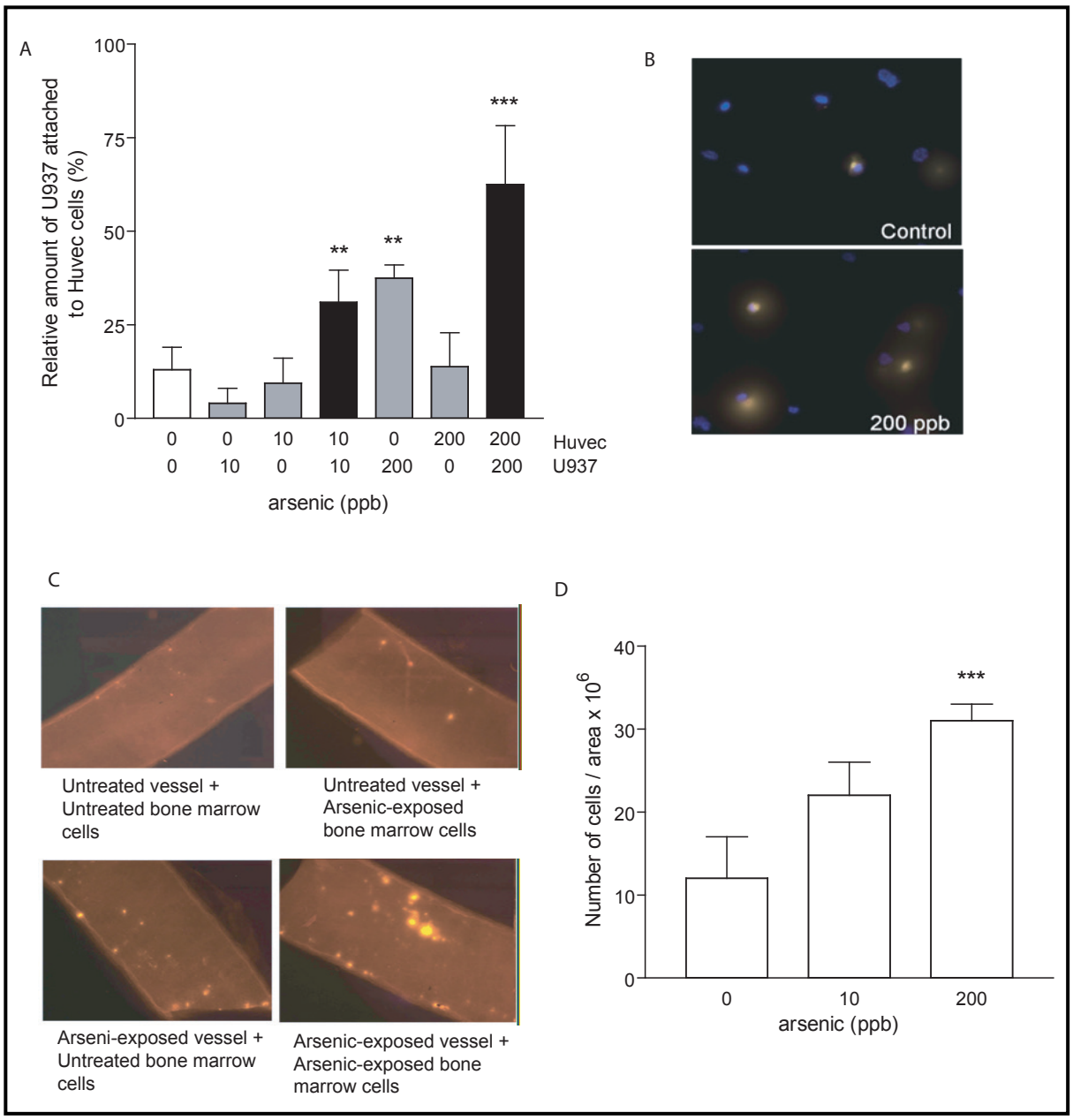

A
Relative amount of U937 attached to Huvec cells (%)
100
75
50
25
0
**
**
***
0 0 10 10 0 200 200
0 10 0 10 200 0 200
Huvec
U937
arsenic (ppb)
B
Control
200 ppb
C
Untreated vessel + Untreated bone marrow cells
Untreated vessel + Arsenic-exposed bone marrow cells
Arseni-exposed vessel + Untreated bone marrow cells
Arsenic-exposed vessel + Arsenic-exposed bone marrow cells
D
Number of cells / area x 10^6
40
30
20
10
0
***
0 10 200
arsenic (ppb)

Figure 4.1. Arsenic induces monocyte adhesion to endothelial cells, with maximal binding achieved following exposure of both cell types.

(A) U937 and/or HUVEC cells (1000 cells/ml) were exposed to arsenic overnight (0, 10 or 200 ppb). U937 cells were fluorescently-labelled and were incubated with HUVEC cells. The non-adhered cells were washed away, and the adherent fluorescent cells were counted. Data are expressed as relative number of U937 over total HUVEC stained cells. **p<0.01; ***p<0.001 B) Representative pictures are shown (40X). C-D) Organ culture of carotid arteries and fluorescently-labelled bone marrow cells where neither, one, or both components were exposed to arsenic trioxide overnight and allowed to adhere to each other for 30 min before being washed. Adherent cells were counted. Representative pictures of 200 ppb arsenic-exposed are shown in C. D) Both components were exposed to arsenic. Data represent ratio ± S.D., $n \geq 3$. **$p < 0.01$; *** $p < 0.001$, compared to unexposed controls.

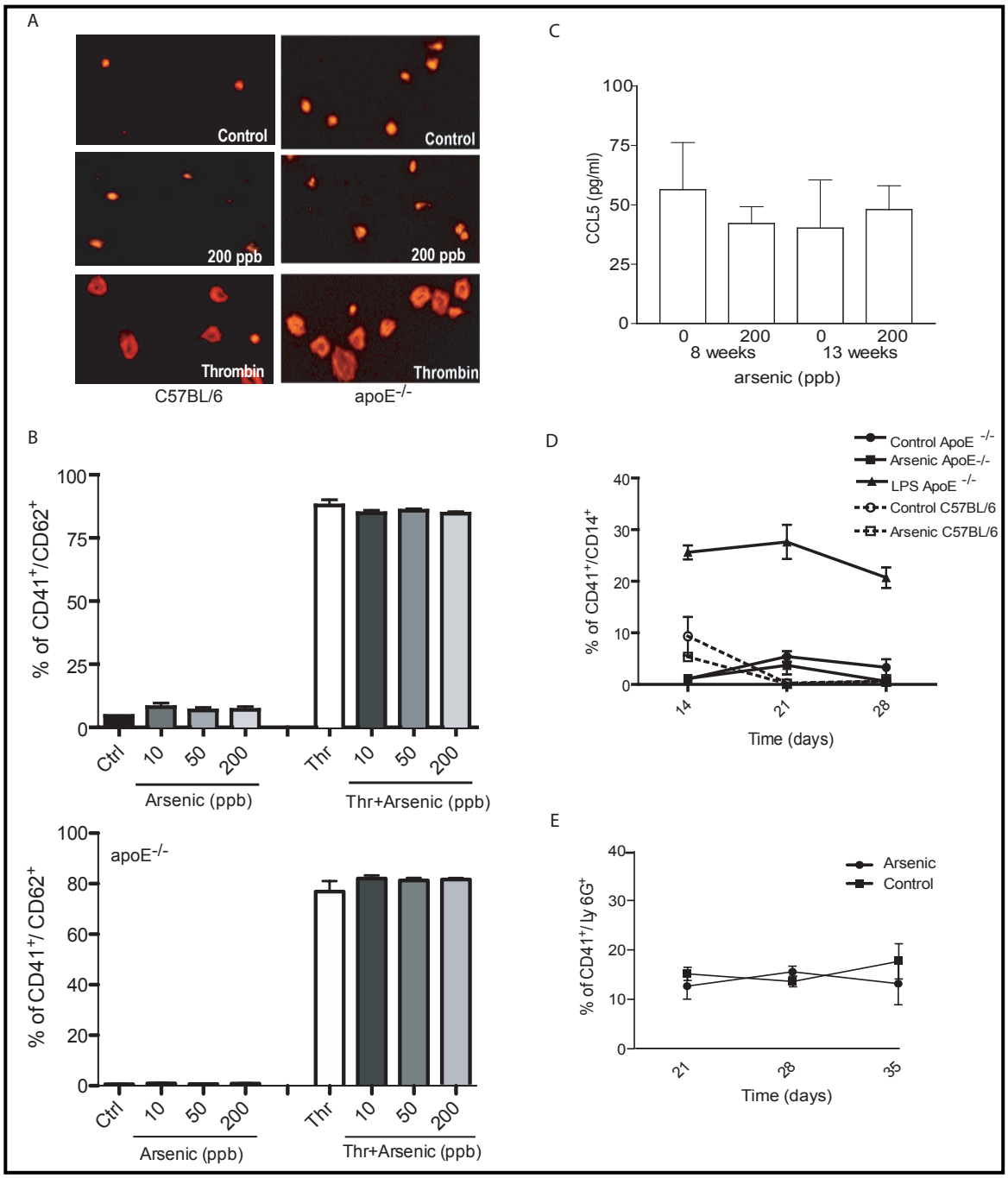

A
Control
Control
200 ppb
200 ppb
Thrombin
Thrombin
C57BL/6
apoE-/-
B
% of CD41+/CD62+
Ctrl
10
50
200
Thr
10
50
200
Arsenic (ppb)
Thr+Arsenic (ppb)
apoE-/-
% of CD41+/ CD62+
C
CCL5 (pg/ml)
0
200
0
200
8 weeks
13 weeks
arsenic (ppb)
D
Control ApoE -/-
Arsenic ApoE-/-
LPS ApoE -/-
Control C57BL/6
Arsenic C57BL/6
% of CD41+/CD14+
Time (days)
E
Arsenic
Control
% of CD41+/ ly 6G+
Time (days)

Figure 4.2. Arsenic does not enhance platelet activation, platelet/monocyte interaction or platelet/neutrophil aggregates formation.

C57BL/6 wild-type and apoE$^{-/-}$ mouse platelets were collected and exposed to arsenic (10, 50 or 200 ppb) and/or thrombin for 5 minutes. Representative pictures of platelet spreading after control, 200 ppb arsenic or 1U thrombin are shown in A (left panels: C57BL/6; right panels: apoE$^{-/-}$). (B) P-selectin (CD62) expression was assessed by flow cytometry (up panel: C57BL/6; down panel: apoE$^{-/-}$). (C) Circulating CCL5 levels were measured in apoE$^{-/-}$ male mice were left untreated or exposed to 200 ppb arsenic for 8 or 13 weeks using an immunoassay kit (multiplex bead-based) on a Bio-Plex 200 (Bio-Rad Laboratories, ON, Canada). Each sample (n = 4) was analyzed in duplicate (technical replicate). (D-E) The platelet/monocyte aggregates (CD14^{+}/CD41^{+}; D) (up panel: C57BL/6; down panel: apoE$^{-/-}$) and the platelet/neutrophil aggregates (Ly6G^{+}/CD41^{+}; E) were followed from day 14 to 28 in the circulation of mice exposed to 200 ppb arsenic. One mouse, as positive control, was treated with LPS for 18 hours before the blood collection. Values are expressed as mean ± S.D., n ≥ 3 animals.

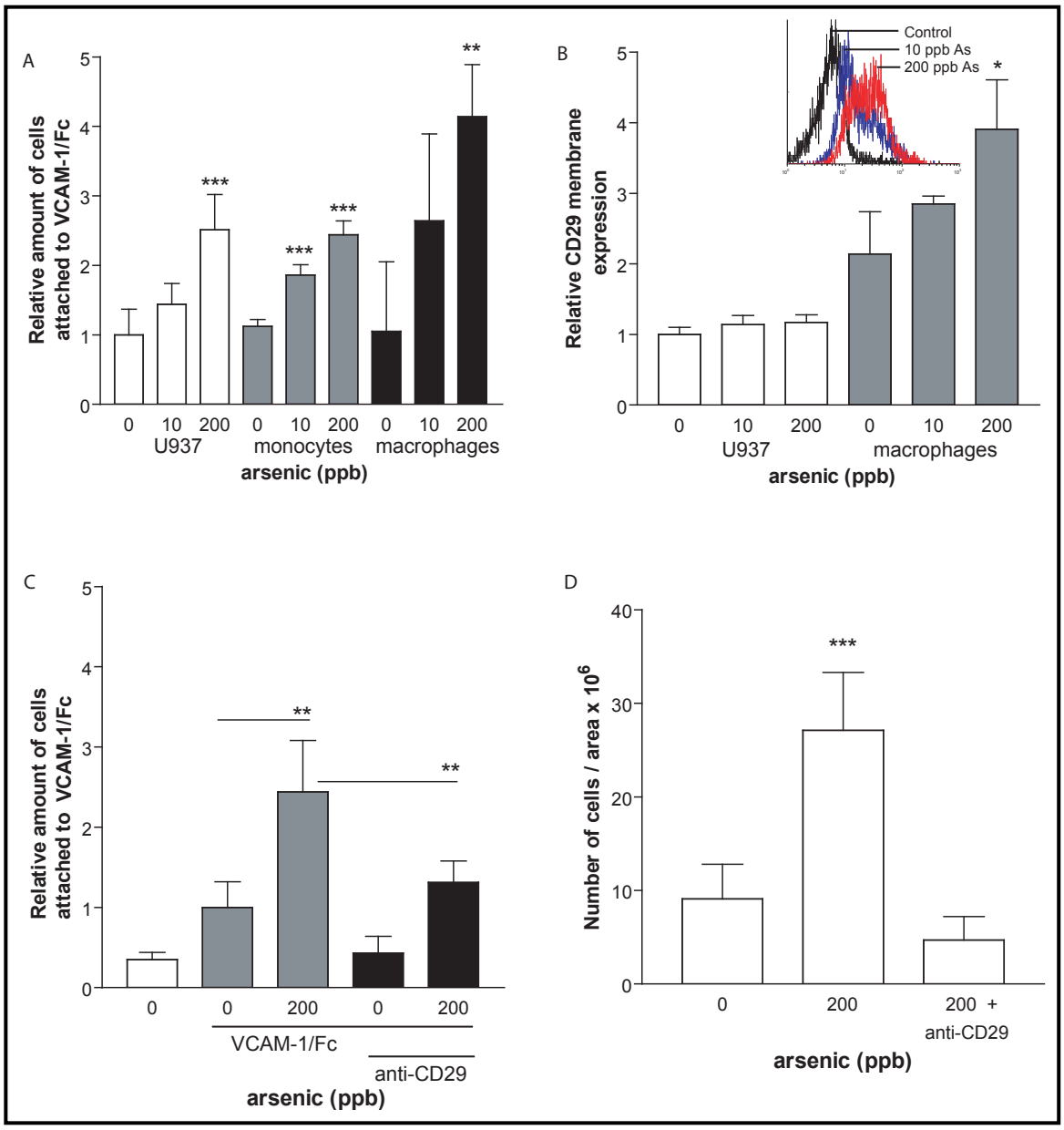

A
Relative amount of cells attached to VCAM-1/Fc
0 10 200 0 10 200 0 10 200
U937
monocytes
macrophages
arsenic (ppb)
B
Relative CD29 membrane expression
Control
10 ppb As
200 ppb As
0 10 200 0 10 200
U937
macrophages
arsenic (ppb)
C
Relative amount of cells attached to VCAM-1/Fc
0 0 200 0 200
VCAM-1/Fc
anti-CD29
arsenic (ppb)
D
Number of cells / area x 10^6
0 200 200 + anti-CD29
arsenic (ppb)

Figure 4.3. Arsenic increases adhesion of mononuclear cells via increased CD29 binding to VCAM-1.

U937 or human PBMC cells (1000 cells/ml) were exposed to arsenic for 72h (0, 10 or 200 ppb) (A). Cells were fluorescently-labelled and incubated on VCAM-1/Fc coated plates. The non-adherent cells were washed away, and adherent fluorescent cells were counted. In B, cellular surface CD29 antigens were detected by flow-cytometry using anti-CD29 antibody. (C-D) CD29 blocking antibody was added to *in vitro* U937 binding assays to VCAM-1/Fc (C) or to *ex vivo* organ cultures with primary mononuclear cells (D). Values are expressed as mean ± S.D., $n \geq 3$. *$p < 0.05$: **$p < 0.01$; ***$p < 0.001$, compared to unexposed controls.

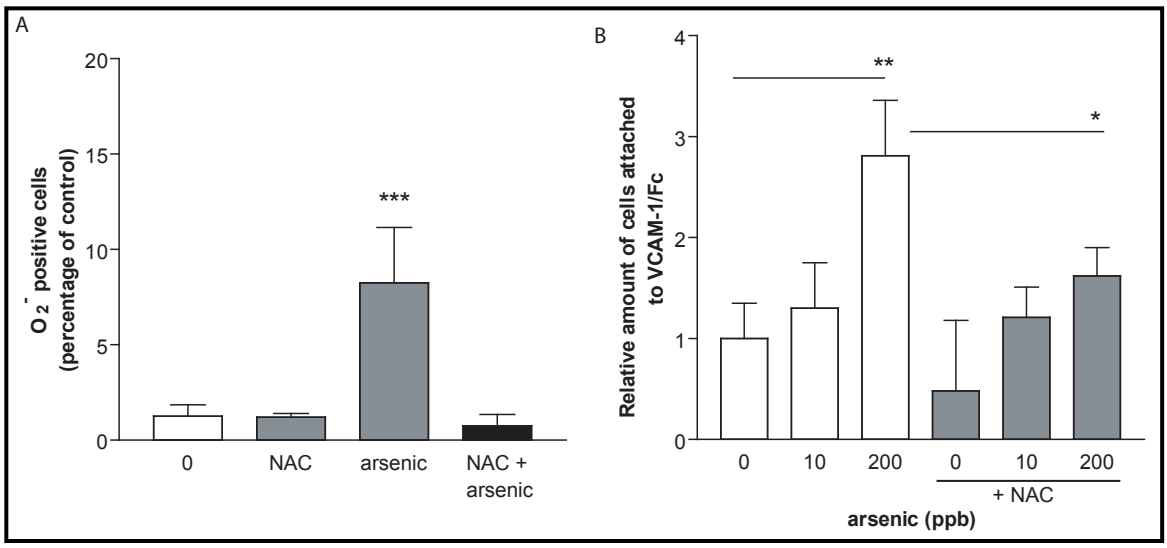

A
20
15
10
5
0
O_2^- positive cells
(percentage of control)
***
0
NAC
arsenic
NAC +
arsenic
B
4
3
2
1
0
Relative amount of cells attached
to VCAM-1/Fc
**
*
0
10
200
0
10
200
+ NAC
arsenic (ppb)

Figure 4.4. Arsenic-induced monocyte adhesion to VCAM-1 is prevented by antioxidant *in vitro*.

U937 cells (1000 cells/ml) were pretreated for 1 h with NAC (1 mM) and then exposed to arsenic for 3h (0, 10, 50 or 200 ppb). Cells were stained with HEt and staining detected by flow cytometry (A). Alternatively, cells were fluorescently-labelled with orange tracker and incubated on VCAM-1/Fc coated plates (B). The non-adherent cells were washed away, and the adherent fluorescent cells were counted. Values are expressed as mean ± S.D., $n \geq 3$. * $p < 0.05$: ** : $p < 0.01$; *** : $p < 0.001$, compared to unexposed controls.

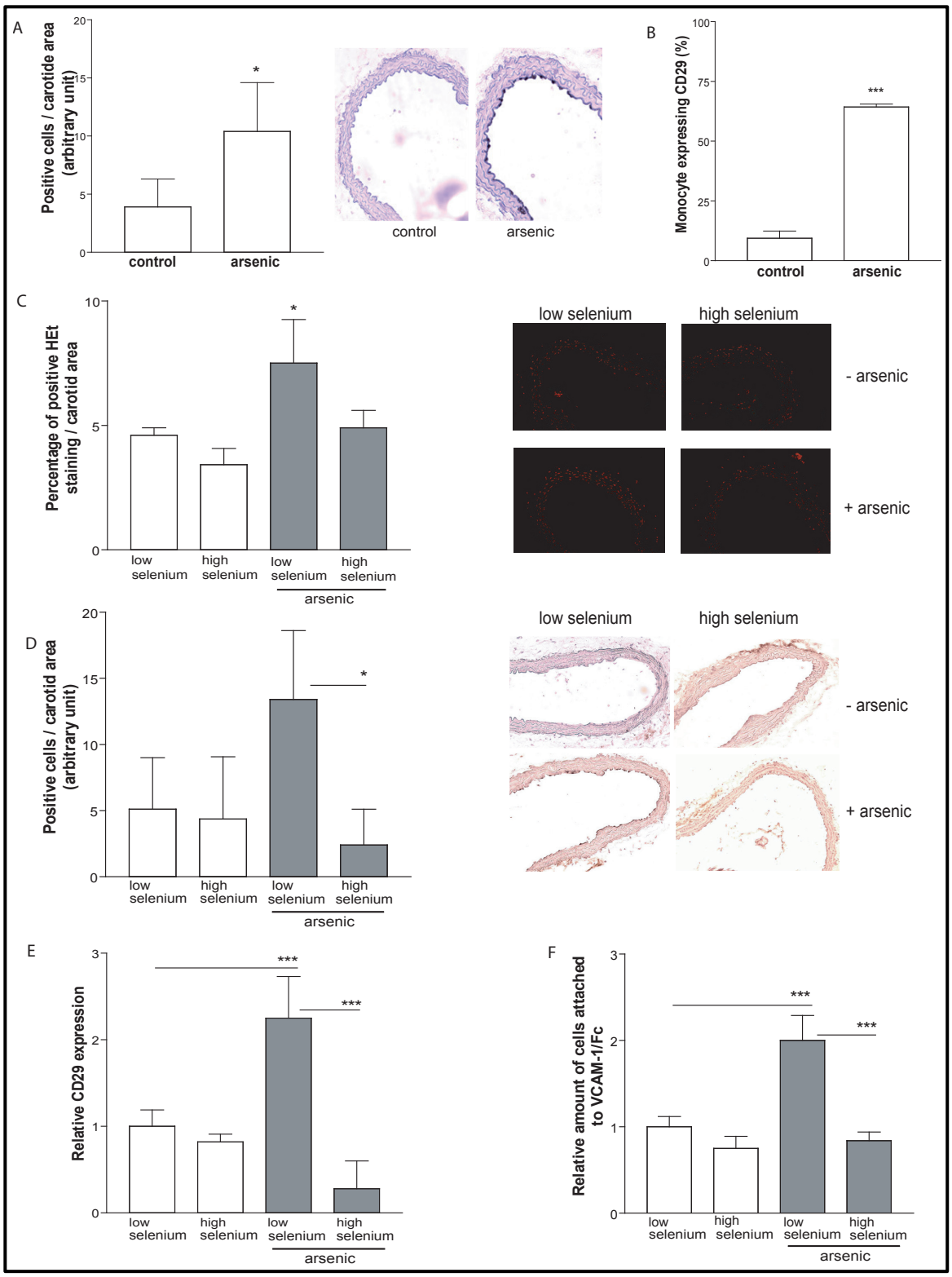
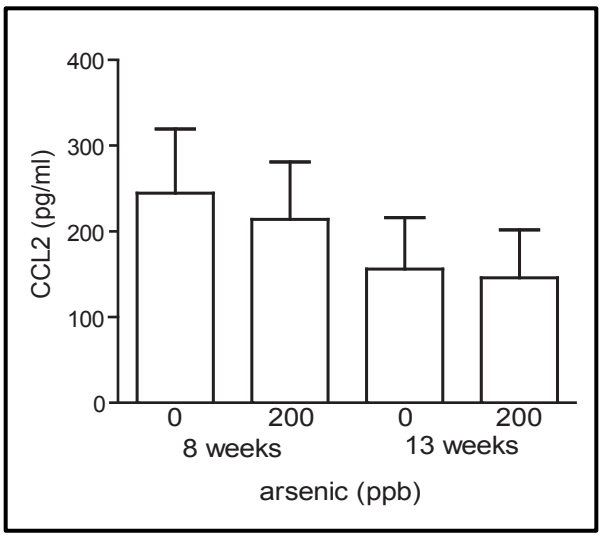

A
Positive cells / carotide area (arbitrary unit)
control
arsenic
B
Monocyte expressing CD29 (%)
control
arsenic
C
Percentage of positive HEt staining / carotid area
low selenium
high selenium
low selenium
high selenium
arsenic
low selenium
high selenium
- arsenic
+ arsenic
D
Positive cells / carotid area (arbitrary unit)
low selenium
high selenium
low selenium
high selenium
arsenic
low selenium
high selenium
- arsenic
+ arsenic
E
Relative CD29 expression
low selenium
high selenium
low selenium
high selenium
arsenic
F
Relative amount of cells attached to VCAM-1/Fc
low selenium
high selenium
low selenium
high selenium
arsenic

Figure 4.5. Arsenic increases adhesion molecule expression *in vivo*, which can be prevented by addition of high selenium diet.

(A-B) In order to evaluate *in vivo* effects of arsenic, five-week-old male apoE$^{-/-}$ mice fed normal rodent diet were exposed to arsenic (200 ppb) for 13 weeks or maintained on tap water. Carotids were stained for VCAM-1 (A) and whole blood was collected and CD29 expression was detected using flow-cytometry (B). (C-F) In order to evaluate ROS involvement in arsenic-induced atherosclerosis, five-week-old male apoE$^{-/-}$ mice were exposed to arsenic (200 ppb) for 13 weeks or maintained on tap water. Mice were fed with low selenium or high selenium chow. Carotids were stained for ROS (C), or VCAM-1 (D). Blood was collected and CD29 expression was detected using flow-cytometry (E), or cells were fluorescently-labelled and incubated on VCAM-1/Fc coated plates (F). Values are expressed as mean ± S.D., n ≥ 3. * $p < 0.05$: ** : $p < 0.01$; *** : $p < 0.001$, compared to unexposed controls.


400
300
200
100
0
CCL2 (pg/ml)
0
200
0
200
8 weeks
13 weeks
arsenic (ppb)

Supplementary Figure 4.1. Arsenic does not increase CCL2 circulating levels.

Circulating CCL2 levels were measured in apoE$^{-/-}$ male mice that received tap water or that were exposed to 200 ppb arsenic for 8 or 13 weeks using an immunoassay kit (multiplex bead-based) on a Bio-Plex 200 (Bio-Rad Laboratories). Each sample (n = 4) was analyzed in duplicate (technical replicate). Values are expressed as mean ± S.D.

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CHAPTER 5

General Conclusions and Future Directions

5.1 Main contributions for the field.

Arsenic exposure is linked epidemiologically with an increased risk of developing atherosclerosis. Our lab has been dedicated to understanding the underlying mechanisms of arsenic-enhanced atherosclerosis. Our research goal was to investigate the role of arsenic biotransformation, an oxidative-methylation reaction catalyzed by As3MT. We utilized mouse models, *in vitro* primary cultures and molecular techniques to dissect the importance of As3MT, to determine whether intermediates of the biotransformation process could be pro-atherogenic, and to identify where *As3MT* expression is required to increase atherosclerosis formation.

Our data show that arsenic biotransformation is an essential mechanism for arsenic toxicity. Importantly, *As3MT* expression is required for arsenic-induced atherosclerosis in both post- and pre-natal exposures. This changes the way we think about arsenic biotransformation, intermediate compounds, and arsenic itself. Trivalent compounds are considered more toxic. However, all methylated arsenicals enhanced plaque formation, which indicates that the relative toxicity is potentially the same for those compounds.

Moreover, the relationship between postnatal arsenic exposure and atherosclerosis requires a full/efficient biotransformation process, because As3MT knockout prevents arsenic-enhanced atherosclerosis. Interestingly, methylated arsenicals also failed in increase plaque formation in that model, which shows that the methylation reaction and not specific arsenic species would be responsible for the increase in atherosclerosis. In addition, deletion of As3MT also protects from arsenic-induced ROS, which was also a surprise, considering that most of the literature attribute the ROS induction to arsenic itself. We demonstrate that As3MT is expressed in multiple cell types that have never been shown before in literature, and more importantly, the As3MT should be expressed in the bone marrow compartment for the pro-atherogenic effects of arsenic to occur. Thus, arsenic has effects on different cell types leading to increase atherosclerosis, which we also have shown in the chapter 4.

In regards to the effects *in utero*, our work validated previous work using 49 ppm arsenic and the apoE$^{-/-}$ mouse model, but at an environmentally-relevant concentration. Beyond that, we also showed that exposure to methylated arsenicals only during development enhances atherosclerosis later in life. Next, As3MT is also required for the arsenic-induced *in utero* pro-atherogenic effects.

Finally, we have shown that arsenic biotransformation is the key mechanism for arsenic enhanced-atherosclerosis in two different exposure scenarios; supporting the hypothesis that single nucleotide polymorphism (SNPs), predictive of altered As3MT enzyme efficiency in humans, could impact arsenic adverse health effects outcome. More studies are required to understand the biotransformation process and its consequences on arsenic pathogenesis.

5.2 Arsenic methylation is required for arsenic-enhanced atherosclerosis.

Methylated arsenicals are intermediate compounds produced during arsenic biotransformation by As3MT [1]. In the chapter 2, we have shown that methylated arsenicals are pro-atherogenic and that they change plaque components to the same extent as sodium arsenite. Importantly, all arsenicals promote plaques with phenotypes more prone to rupture. To our knowledge, this is the first report that investigated the toxicities of methylated arsenicals in a full animal model of cardiovascular disease. Because the methylated arsenicals are pro-atherogenic (Figure 2.1), we decided to investigate the impact of arsenic biotransformation on the outcome. Thus, we developed a unique double knockout mouse model that forms atherosclerosis and has a severe impairment in the arsenic metabolism reaction. In fact, apoE$^{-/-}$ /As3mt$^{-/-}$ mice are resistant to arsenical-enhanced, but not western diet-induced, atherosclerosis (Figure 2.2). Hence, As3MT expression is driving the atherosclerosis, which supports the hypothesis that the biotransformation is enhancing arsenic toxicity, rather than producing less toxic compounds. The previous statement is not an isolated phenomenon in toxicology. Several compounds are well described in literature, where the metabolic intermediates are more toxic than the parent compounds. For example, it is widely accepted that polycyclic aromatic hydrocarbons develop their toxic effects only after a series of biotransformation in the liver [2].

Interestingly, we shed light onto the impact and importance of As3MT to arsenic related effects, because As3MT is also required for ROS production by arsenic. Figure 2.3 clearly shows that *As3MT*

knockout prevents ROS induction in the animals that were exposed to sodium arsenite and methylated arsenicals. *In vitro* experiments with primary cells support this result. This finding is groundbreaking, because arsenic-induced ROS was thought to be generated from arsenic-containing compounds and their interactions with cellular components, such as mitochondria [3, 4] and NADPH oxidase, with MMA III potentially being the most reactive [5]. ROS production has not been linked to the biotransformation process, beyond creation of these methylated intermediates. Our data support a role for As3MT in addition to generation of MMA III, because deletion of As3MT prevented MMA III-induced ROS in vivo (Figure 2.3) and in vitro (data not shown). Indeed, cells that had a reduced capacity for methylation were resistant to arsenic-induced oxidative DNA damage; while, methylation-efficient cells acquired malignant features faster than deficient one [6]. This work demonstrated that arsenic methylation may be required for DNA damage and an acquired cancer phenotype in cell lines. On the other hand, the same group has shown that MMA III induces oxidative damage in cell lines that have severely impaired biotransformation [7], whereas in our model even that compound failed to increase ROS in the DKO animals. Herein, we should caution that the diversity of models might impact the observed outcome, because the previous studies have used mainly human cell lines that have deficient As3MT and not a knockout of the enzyme. Likely, As3MT null cells would be more resistant than the deficient ones that could still have small amount of basal methylation. Likely, the biotransformation reaction is required for any arsenical to induce ROS, though the mechanism is yet-to-be identified.

The arsenic biotransformation process is thought to occur mainly in the liver. However, we have shown that the plaque-resident cells express *As3MT* (Figure 2.4). Moreover, we determined that As3MT expression is necessary in the bone marrow compartment to increase plaque formation (Figure 2.5, A). This represents a shift from the paradigm where arsenic intermediate effects were believed to happen after liver-centric biotransformation. Nevertheless, we could not define the cell type that drives the atherosclerosis outcome.

The correlation between efficiency of arsenic biotransformation and disease outcome has been assessed in several epidemiological studies [8-17]. Frequently, %iAs, %MMA, %DMA, and MMA/iAs and MMA/DMA ratios are assessed in urinary human samples. MMA/iAs and MMA/DMA ratios are indicative of efficiency of the first and second methylation steps, respectively. However, several situations may

influence the biotransformation efficiency, such as nutritional status, individual genotype and exposure concentration, which would impact directly the metabolite concentration in urine. For example, increase in folate intake was correlated with more DMA in urine [18]. Another study reported that highly-exposed populations may have an incomplete methylation process, which has been hypothesized to result from As3MT saturation, leading to a decreased second methylation event or arsenic inhibits As3MT function [8]. This explanation was recently challenged by data showing that As3MT binds to arsenic and only dissociates after the last round of methylation [19], suggesting that the enzyme could become saturated at high concentrations, however it should not decrease a particular methylation event. We could not find reports that described As3MT inhibition by excess of arsenic. Increase in %MMA in the urine is correlated with enhanced carotid intima thickness, a marker of early atherosclerosis [9]. Indeed, MMA is generally used as biomarker that would predict more risk of adverse health effects [20]. We have shown that As3MT knockout protects against MMA-induced plaque formation and ROS. Thus, our result may help to understand that, actually, more MMA in the urine doesn't mean that this compound itself is causing atherosclerosis. A yet-to-be defined mechanism could explain why an inefficient enzyme is impacting the outcome. Moreover, functional tests with described *As3MT* SNPs might even predict which individuals are more at risk of arsenic adverse health effects.

As3MT functions beyond arsenic methylation have been suggested in literature, however there are no other roles defined for the enzyme. As3MT is a methyltransferase, a superfamily of enzymes that have several functions, from DNA methylation to post-translational modification of histone and other proteins. Interestingly, some animals, such as marmoset and chimpanzees, do not methylate arsenic [21, 22], but do express As3MT. Then what would its function be in those species? Recently, a genome wide association study in mental health has revealed association of polymorphisms in the locus of 10q24 chromosome [23], where As3MT is located. Moreover, an isoform of As3MT was related to schizophrenia, apparently this isoform does not methylate arsenic [24]. Therefore, it is fair to speculate that this enzyme would have broader roles than only methylation of arsenic. More studies are necessary to understand the As3MT functions besides biotransformation of arsenic. Although no major alterations have been reported in the $As3MT^{-/-}$ mice [25], KO mice may develop disease later or under stress. We observed that $As3MT^{-/-}$ mice have more abdominal fat than the WT, which was later confirmed by personal

communication with Dr. David Thomas from US EPA. Mechanisms that support this phenotype are still under investigation. Indeed, WT and As3MT$^{-/-}$ had different patterns of plasma and urinary metabolomics [26]. As3MT$^{-/-}$ mice may change utilization of the major methyl donor in cells, S-adenosymethionine (SAM), because some of the altered metabolites were products or substrates of methylation reactions that use SAM [26]. As3MT is dependent on SAM as a methyl donor; further As3MT is not the only enzyme that uses that methyl donor. DNA methyltransferase enzymes that are responsible to directly methylate DNA are also dependent on SAM. Thus, DNA hypomethylation has been related to SAM depletion due to arsenic methylation. However, SAM levels measured in the blood and DNA methylation were not correlated with arsenic metabolites [27]. In fact, DNA methylation and arsenic biotransformation consume very little of the total level SAM [28]. Thus, it is unclear how epigenetic modification relates to SAM and arsenic.

5.2.1 Future directions and proposed experiments for post-natal exposures.

Our project opens several new avenues of investigation. To unveil where As3MT is important in the atherosclerotic lesion, further *in vivo* model studies are required. Arsenic-induced atherosclerosis could be compared between animals expressing Cre in different cell types to define the relevant contribution of As3MT expression in hepatocytes versus macrophages, endothelial cells, and smooth muscle cells in As3MT floxed in mice. As3MT floxed mice could be generated and then bred with apoE$^{-/-}$ mice and mice expressing tissue-specific Cre recombinase. Commercially-available Cre mice could be used to dissect specific cellular contributions: albumin-Cre (hepatocytes), LysM-Cre (macrophages), Tie2-Cre (endothelial cells) and transgelin (smooth muscle protein 22-alpha).

Our data shows that As3MT expression is required for arsenic-induced ROS, although ROS is not expected to be a byproduct of the arsenic methylation reaction. What produces ROS during arsenic methylation? One would predict the most reactive methylated arsenical, MMA III, causes ROS. However our ex vivo data in DKO mice (Figure 2.4) and a preliminary data in As3MT$^{-/-}$ macrophage cultures (data not shown) demonstrate that MMA III also failed to increase ROS when As3MT is deleted. In order to address the molecular mechanism of arsenic methylation inducing free radicals, we could compare gene/protein expression and organelle function in WT and As3MT$^{-/-}$ mouse embryonic fibroblasts.

As previously described, differences in methylation in humans are related with disease outcome. Perhaps, ROS generation is linked to the rate or efficiency of the methylation reaction. To test this, we could reconstitute the As3MT$^{-/-}$ fibroblast with wild-type human As3MT or the described M287T SNP variant that increases methylation efficiency [29]. Such experiments would be key to understanding As3MT SNPs in arsenic related effects; moreover *in vitro* modeling could be used to predict arsenic related effects of described SNPs in population.

Alternatively, we are interested in the other functions of As3MT, besides arsenic methylation. Recently, another arginine methyltransferase (RMT) was identified, suggesting there are other RMTs beyond the classical PRMTs [30]. We hypothesized that As3MT would be an arginine methyltransferase. Indeed, the lab has preliminary results showing a different pattern of methylation on symmetrical arginine in liver extraction (data not shown). Identification of targets for As3MT-mediated arginine methylation could be identified by immunoprecipitation (IP) with anti-symmetrical arginine antibody followed by mass spectrometry in liver lysates from WT and As3MT$^{-/-}$ mice Target specificity could be confirmed *in vitro* by methylation assays using radiolabeled-SAM as the methyl donor and target proteins.

5.3 Arsenic methylation enhances plaque formation later in life after prenatal exposure

Recently, the US National Academy of Sciences has emphasized the necessity to understand the later life effects of arsenic after exposure during development [31]. An increase in young adult mortality [32] and respiratory diseases in children [33] was observed after an accident in Antofagasta, Chile, where the population was exposed to 12 years of high arsenic concentration in the water. Thus, some subjects were exposed only during development, which may increase their susceptibility to develop diseases later in life. Previous work in the literature has shown that high concentrations of arsenic increase atherosclerosis later in life after prenatal exposure [34]. Herein, we were interested in assessing whether *in utero* exposure to environmentally-relevant concentrations of arsenicals had the potential to enhance atherosclerosis later in life. In light of our previous data showing an essential role for As3MT in post-natal arsenic exposure and atherosclerosis, we also wanted to determine whether atherosclerosis induced by in utero arsenic was also dependent upon As3MT expression.

Arsenic and arsenicals pass through the placenta, thus the fetus is exposed to similar concentrations of arsenic as the mother [35, 36], and DMA is the form commonly found in the cord blood. Similar to humans, arsenic concentrations in the mouse embryo is comparable to the dams [37]. Therefore, arsenic is a risk factor for infants during development. Regardless of the exposure, arsenic concentration in the breast milk is considerably low [35, 38, 39]. Epidemiological studies have started to include assessment of the maternal methylation capacity when investigating arsenic effects early in life. Recently, maternal urinary MMA negatively correlated with birth weight and gestational age in a cohort from Mexico [40]. The same group investigated the impact of maternal genotype on birth outcomes. Interestingly, five out of seven SNPs were associated with maternal urinary arsenic concentration and alleles for one SNP were correlated with placenta weight and marginally significant for birth weight [41]. In the chapter 3, we evaluated the pro-atherogenic properties of sodium arsenite and methylate arsenicals after *in utero* exposure. After weaning, litters were kept for 13 weeks in tap water; surprisingly inorganic arsenic and methylated arsenicals increase plaque formation later in life in both males and females (Figure 3.1). To some extent, the exposure also alters plaque components, which might be a risk factor for complications due to plaque rupture. Surprisingly, we did not observe increased ROS production in the vessels of mice exposed *in utero* to arsenic (Figure 3.3). This contrasts with what we observed in post-natally-exposed mice. Our data do not rule out a role for ROS at earlier time points, but do indicate that a sustained level of ROS is not required for the pro-atherogenic effects of arsenic. In fact, high levels of ROS may have occurred during the exposure period. Oxidative stress in the placenta and cord blood has been reported as a consequence of arsenic exposure in humans [42, 43].

Because we have observed that arsenic methylation is required for arsenic-increased atherosclerosis in our post-natal model (Figure 2.2), we decided to investigate the impact of biotransformation during prenatal exposure. In fact, *As3MT* deletion protects the progeny from arsenic enhance atherosclerosis later in life (Figure 3.4). To our knowledge, this is the first report in literature demonstrating that biotransformation impacts the outcome later in life after prenatal arsenic exposure.

Arsenic effects have been reported in a transgenerational carcinogenesis model, where males and females offspring from dams exposed to high arsenic concentrations developed tumors in several organs, such lung, adrenal and liver [44]. The same group has reported that mice that were previously

exposed *in utero* have enhanced tumor incidence, when they are concomitant chronically exposed to DMA [45] or tumor promoters [46] after birth or inorganic arsenic during whole life [47]. In addition, another group exposed pregnant mice (F_0) to arsenic, but the subsequent generations (F_1 and F_2) were not. Interestingly, only the F_2 males (progeny from F_1 male mice) had higher incidence of tumors than the control, while the females did not differ [48]. Although, a very high arsenic concentration was used in both studies, the results are interesting, because it shows that arsenic effects are not constrained only to the subjects that are constantly exposed. Recently, a literature review has discussed the necessity of relevant concentrations, because most of the studies, mainly in carcinogenesis have used extreme concentrations and had inconsistent findings [49]. Our data showed that a non-carcinogenic effect is imprinted in both sexes after arsenic exposure, and importantly with an environmental-relevant concentration.

Our data showing that *in utero* exposure to arsenicals causes sustained effects suggests that epigenetic programming may be induced early during arsenic exposure. Indeed, arsenic has been reported to disrupt epigenetic code. Chronic As(III) and MMA(III) exposure in urothelial cells alter DNA methylation at specific promoter regions that were associated with a decrease in H3 acetylation and gene expression during malignant transformation [50]. Moreover, aberrant DNA hypermethylation and histone modification result in silencing of genes encoding zinc finger proteins, one of the largest family of transcription repressors [51]. Hypo-acetylation of histone H3K9 was related with cognitive impairment in adult mice that were exposed to 100 ppb *in utero* [52]. In fact, histone modifications may play a role in the progression of atherosclerosis [53, 54]; hence it would be interesting to assess epigenetic changes in our model.

With regards to humans, emerging epidemiological studies are addressing arsenic exposure, gene expression and epigenetic alterations early in life, however, data are inconsistent and analysis of samples are limited due to techniques high cost. Gene expression from cord blood of newborns that were exposed *in utero* exhibited upregulation of inflammatory genes, mainly by activating the NF-κB signaling cascade [55]. In a cohort from Bangladesh, moderate arsenic exposure was associated with an increase in LINE-1 methylation in leucocyte DNA from mother and cord blood [56]. Another cohort from US with over 150 mother-infants has shown increases in DNA methylation in CpG islands in total cord blood cells, which correlated with increased arsenic exposure. The same study reported $CD8^+$ T lymphocyte

enhancement in the cord blood that was associated with urinary inorganic arsenic [57]. Interestingly, a study from Mexico has coupled DNA methylation and mRNA expression from cord blood cells to understand whether the alterations in DNA methylation are functionally modifying transcription. Most genes with altered methylation did not have altered mRNA expression related with urinary arsenic, an important piece of information showing that not all observed changes would be functional. The authors recommended that the subsequent studies should focus on CpG islands instead of global DNA methylation [58]. Recently, a cohort comparing placenta, umbilical artery, and human umbilical vein endothelial cell (HUVEC) has observed CpG island hypermethylation in placenta, hypomethylation in umbilical artery and no alteration in HUVEC after prenatal exposure to arsenic. Although no functional analysis was performed, the authors discussed that this hypomethylation may be of particular importance, because CpG island hypomethylation has been reported in atherosclerosis lesions [59]. Definitely, those different methylation patterns still need to be associated with biological effects, metabolism and health outcomes.

MicroRNA (miRNA) are also part of the epigenetic mechanism that alters gene expression. miRNA are non-coding RNA that regulate post-transcriptional gene expression [60] and when upregulated, can lead to cancer, respiratory diseases and diabetes. In a model of prostate cancer, arsenic exposure decreases several microRNA that was associated with increase of oncogene from the RAS family[61]. Recently, low to moderate maternal arsenic exposure has been associated with increased miRNA expression in newborn cord blood [62]. Most of the miRNA changed were related with inflammatory responses, specifically in silico testing predicted that those miRNA may inhibit genes related with innate and adaptive immune systems [62]. Of the miRNAs highly expressed *in utero* after arsenic exposure, several overlap with the miRNA related with atherosclerosis described in a recent review [60]. For example, miRNA-126 was overexpressed in the cord blood. However, an upregulation in this miRNA in atherosclerosis is probably a protective mechanism [60]. Therefore, the data are still confusing and more studies are required to define the impact of miRNA modulation after arsenic exposure *in utero*. Perhaps, our model may be useful tool to experimentally assess miRNA alterations, identifying ones that are sustained later in life

Finally, our data have relevance to public health and intervention strategies for arsenic removal. Our data indicate that in utero exposure alone is enough to program a pro-atherogenic phenotype. Therefore, public health policy should be aware that mitigation of arsenic exposure might not be sufficient to reduce the burden of disease, and the risk of arsenic exposure may be much greater and complex than previously thought. Hence, research that elucidates the mechanism of arsenic exposure and later health effects should be supported, discovering biomarkers to predict susceptibility.

5.3.1 Future Directions and Proposed experiments for pre-natal exposures.

While our data show increased plaque formation after prenatal exposure, we did not uncover the mechanism(s) involved. Thus, we have several unanswered questions that should be addressed in the future, including: Is epigenetic alteration driving the phenotype? Which cell type(s) is involved?

We propose that As3MT-dependent epigenetic changes are important for enhanced atherosclerosis. Global DNA methylation in the plaque site has been reported, moreover several miRNA are differently modulated during atherosclerosis formation. We have described that arsenic directly target macrophages and endothelial cells. Therefore, DNA methylation and miRNA expression changes could be measured in those cells sorted from the apoE$^{-/-}$ or DKO plaque/arteries of the *in utero* exposed animals. Functional validation has to be performed to confirm the participation of these deregulations on arsenic-induced atherosclerosis later in life. This approach would be interesting to identify persistent alterations that might be responsible for the outcome. Moreover, it would guide epidemiological studies to refine biomarker research.

5.4 Other pro-atherogenic mechanism of arsenic.

The main focus of the present thesis was to evaluate the impact of arsenic methylation in arsenic-enhanced atherosclerosis. However, arsenic effects are multifactorial. Thus, to better understand the pro-atherogenic effects of arsenic, we have investigated several mechanisms involved early in the development of atherosclerosis, such as cell-to-cell interaction, cell activation and cytokine production.

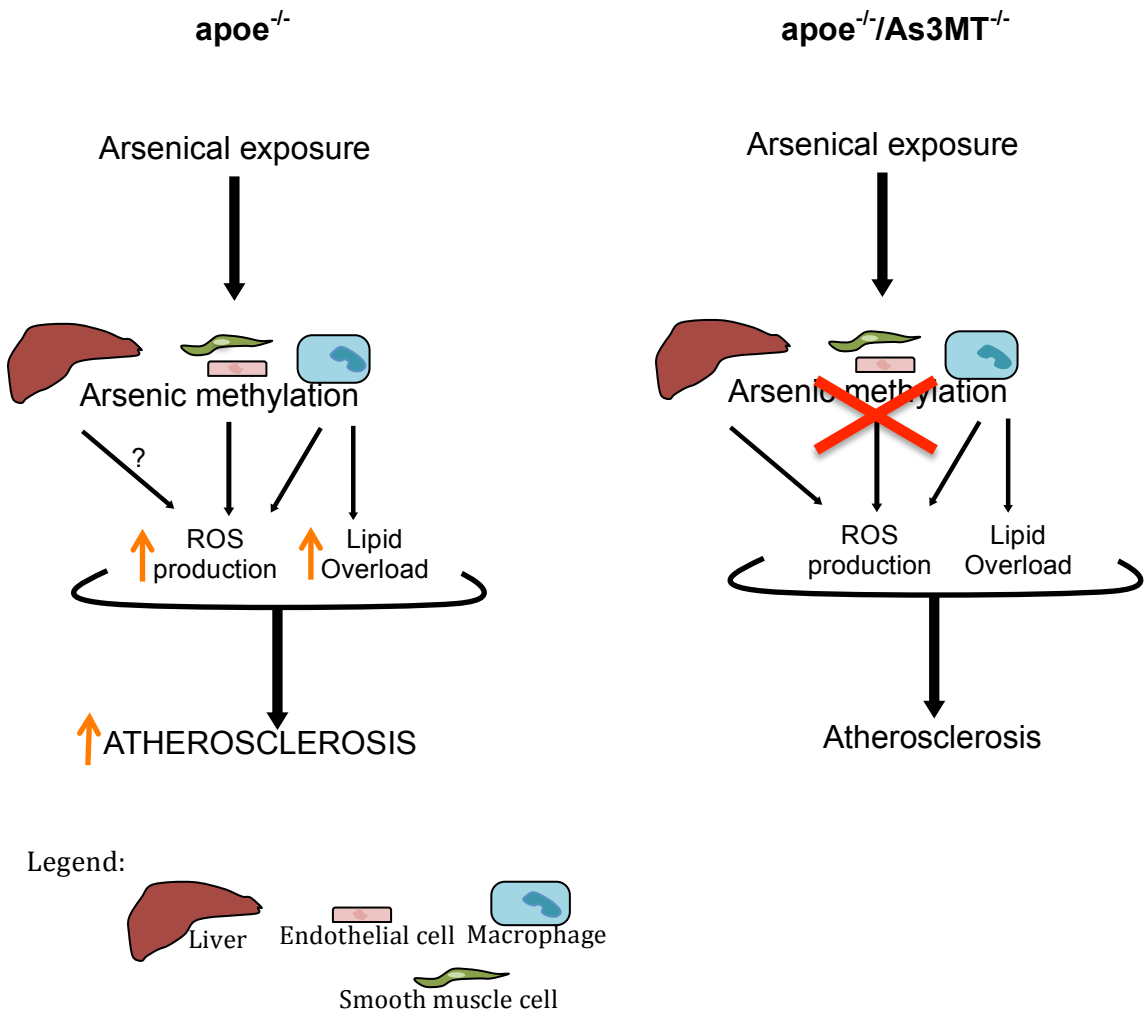
As we have described, one of the main mechanisms of atherosclerosis initiation is flow disturbance along with monocytes adhesion to endothelial cells. Hence, in chapter 4, we have described

that arsenic effects happen at the beginning of atherosclerosis process; increasing the interaction between monocytes and endothelial cells towards VCAM-1 expression, which is mediated by ROS production and blunted after antioxidant administration *in vivo* and *ex vivo*. Our results demonstrated that arsenic-enhanced atherosclerosis does not involve increases in platelet activation or leucocyte-platelet aggregates. In discordance with the literature [63, 64], we did not find evidence for arsenic activation of platelets, even after thrombin stimulation. This may be due to high concentrations of arsenic used in these studies. Platelets are known to participate at the beginning of the atherosclerotic process, recruiting leucocytes, and facilitating their adhesion to the endothelium [65, 66]. Our results that demonstrate no effects of arsenic on platelets stress the arsenic specificity effects enhancing leucocytes/endothelial cells interactions.

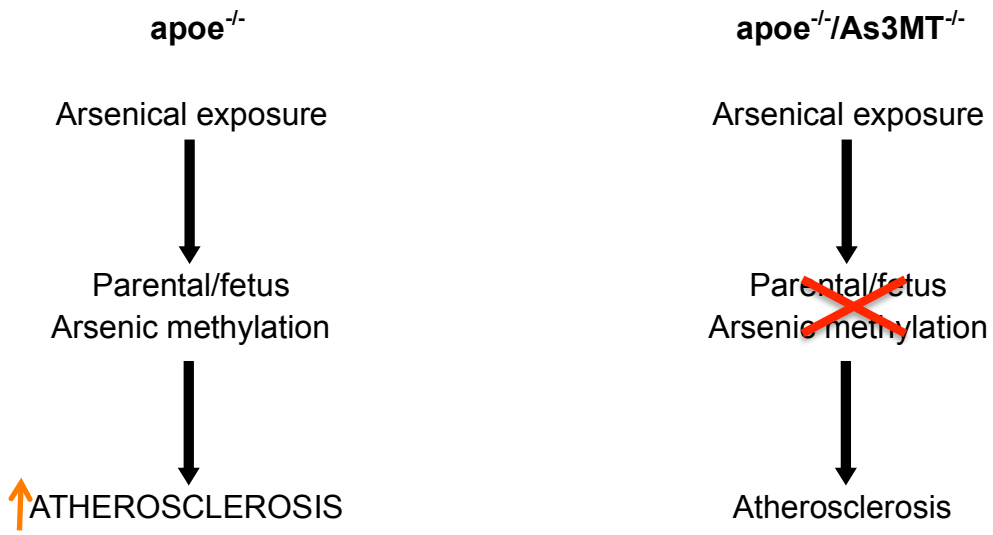
Arsenic has been reported to affect endothelial cells [67-69] and macrophages/monocytes[70-74]. We add to that showing that arsenic specifically alters monocyte and endothelial cell homeostasis, but also changes their interaction, which contributes to atherosclerosis formation. Our further characterization shows the importance of oxidative stress in the mechanism of that interaction. Mice fed with selenium-rich diet have a reduced arsenic-activated endothelium and fewer cells attached to VCAM-1. Selenium is an essential micronutrient that acts as a co-factor for antioxidant enzymes, such as thioredoxin reductase and GSH-peroxidase [75]. Selenium also enhances arsenic excretion, because it forms a complex that is excreted by hepatobiliary system [76]. Therefore, it would be expected that selenium supplementation protects from arsenic-induced damage. Indeed, a diet rich in selenium prevents arsenic-enhanced atherosclerosis in our apoE$^{-/-}$ mouse model [77]. Perhaps dietary strategies may be developed in places where the arsenic mitigation in the drinking water is not effective.

5.5 Graphic Model

5.5.1 Arsenic methylation is required for arsenic-induced atherosclerosis



5.5.2 Prenatal arsenic exposure enhances atherosclerosis via an As3MT-dependent mechanism



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