THE ROLE OF DIETARY FOLATE INTAKE IN METABOLISM OF ARSENIC IN WILD-TYPE C57BL/6N MICE AND C57BL/6N MICE CARRYING THE HUMAN *AS3MT* GENE

Madison Miller

A thesis submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Nutrition in the Gilling's School of Global Public Health.

Chapel Hill
2022

Approved by:

Mirek Styblo

Beverly Koller

Sergey Krupenko

ABSTRACT

Madison Miller: The Role of Dietary Folate Intake in Metabolism of Arsenic in Wild-Type C57BL/6N Mice and C57BL/6N Mice Carrying the Human *AS3MT* Gene
(Under the direction of Mirek Styblo)

Chronic exposure to inorganic arsenic (iAs) is a public health concern and is associated with type 2 diabetes. iAs metabolism consists of methylation reactions catalyzed by arsenic methyltransferase (AS3MT) in most mammals. In humans, folate intake has been shown to affect iAs metabolism efficiency.

Mouse models are commonly used to study iAs exposure, but mice are more efficient at metabolizing iAs than humans. This study examined a new mouse model carrying human *AS3MT* to better represent human iAs metabolism. We also assessed the role of folate in iAs metabolism and development of diabetic phenotypes in humanized and wild-type mice fed a folate deficient, adequate, or supplemented diet. We found that the humanized mice are a suitable model for human iAs metabolism but do not exhibit the same effects of folate intake on iAs metabolism seen in humans. Folate intake did not alter iAs metabolism efficiency in mice of either genotype.

ACKNOWLEDGEMENTS

I would like to thank Dr. Mirek Styblo for providing me with guidance over the past three years and for always having an open door. Your kindness and support were significant factors making my experience conducting research so positive. I would like to thank Dr. Christelle Douillet for mentoring me and teaching me a wide range of scientific techniques, as well as encouraging me develop a more scientifically questioning mind. Your patience and support have been valuable to me and has strengthened my love for science and research. Thank you also to Dr. Beverly Koller and Dr. Sergey Krupenko, for their guidance in navigating my Masters research over the past year. To the members of the Styblo lab I owe an immense amount of appreciation for creating a collaborative and warm environment. Last, I want to thank my family and friends for understanding how important my research has been to me over the last three years. Thank you for always being willing to listen to my ramblings.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AS3MT	Arsenic (+3 oxidation state) methyltransferase
FA	Folate adequate
FBG	Fasted blood glucose
FD	Folate deficient
FPI	Fasting plasma insulin
FS	Folate supplemented
HOMA-IR	Homeostatic model assessment for insulin resistance
HOMA-B	Homeostatic model assessment for beta cell function
Hu	Humanized-*AS3MT*
iAs	Inorganic arsenic
ppb	parts per billion
tAs	Total arsenic
WT	Wild-type

CHAPTER 1: INTRODUCTION

Inorganic Arsenic

Inorganic arsenic (iAs) is a toxic metalloid found in many minerals and ores. Leaching of iAs from these sources leads to high iAs concentrations in groundwater. iAs has been found to be dangerous to human health in both acute and chronic doses due to its role as a carcinogen and diabetogen (US EPA, 2015; WHO, 2019). Because of its carcinogenic effects, the US Environmental Protection Agency (EPA) and the World Health Organization (WHO) both recommend a limit of 10 ppb in drinking water. The US Food and Drug Administration (FDA) has set limits in some foods, such as 100 ppb for infant rice cereal and 10 ppb in apple juice (US FDA, 2020).

In most mammals, including humans, the metabolism of iAs involves a series of methylation reactions, first forming monomethyl-As (MAs), then dimethyl-As (DMAs), in which arsenic is found either in the +5 or +3 oxidation state. Both of these methylation steps are catalyzed by arsenic (+3 oxidation state) methyltransferase (AS3MT) or its species-specific orthologs (Lin et al., 2002; Thomas et al., 2007). There are two mechanisms proposed to explain how arsenic is methylated, both of which include pathways for the formation of pentavalent and trivalent arsenicals. The first scheme (**Figure 1**), proposed by Challenger, involves a series of alternating oxidative methylations and reductions, in which arsenic metabolites alternate between the +3 and +5 oxidation states (Challenger, 1947). The second scheme (**Figure 2**), from newer

evidence, proposes that trivalent species undergo methylation without oxidation, though these arsenicals can undergo spontaneous oxidation to the pentavalent forms (Stýblo et al., 2021). All arsenical species are toxic, with the trivalent intermediates MAs^{III} and $DMAs^{III}$ as the most toxic. Thus, while the methylation of iAs is a detoxification process by facilitating the clearance of iAs, it is also an activation pathway due to the formation of highly toxic trivalent metabolites (Stýblo et al., 2021; Thomas et al., 2001). There have been many factors shown to affect the efficiency of iAs methylation, including intake of the nutrients involved in one carbon metabolism (OCM) such as folate (Sijko & Kozłowska, 2021).

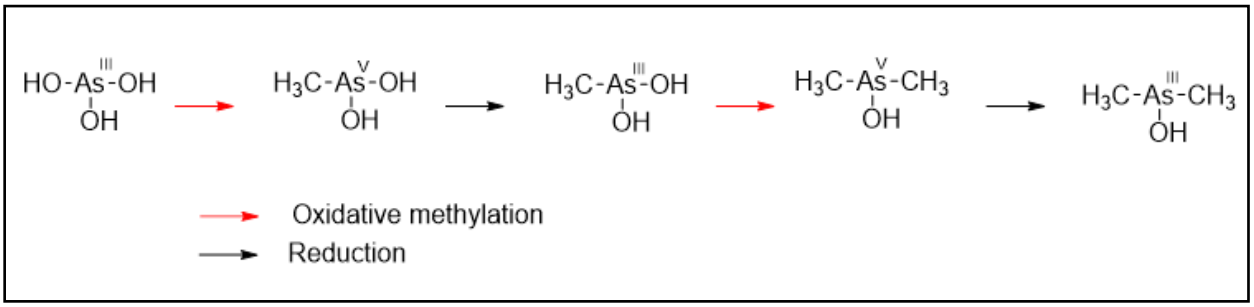

Figure 1: Challenger scheme of oxidative methylation of arsenic

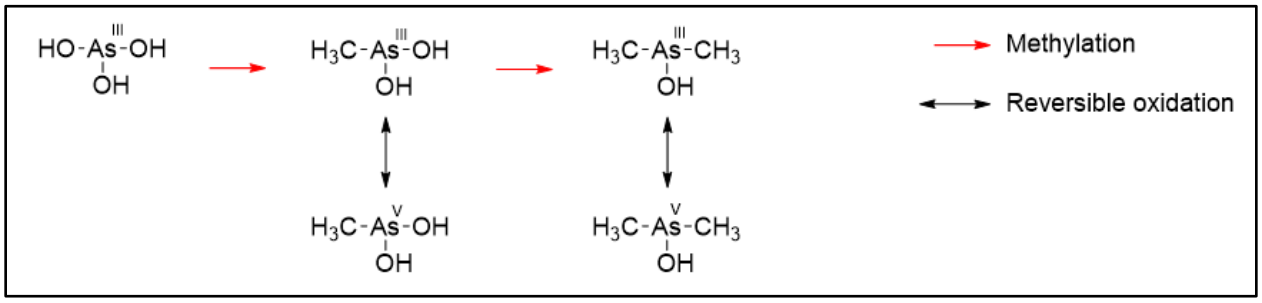

Figure 2: Alternate scheme of arsenic methylation with secondary oxidations

Folate

Folate is a term encompassing a group of pteroylglutamate compounds with vitamin B9 activity. The folate structure consists of a pterin ring, para-aminobenzoic acid, and a chain of

glutamate residues. The recommended dietary intake of folate is 400 µg of dietary folate equivalents (DFE) per day, with a higher intake (600µg) recommended for pregnant women (Institute of Medicine (US), 1998). Folate is an essential micronutrient that cannot be synthesized by mammalian cells and must be consumed by mammals to achieve adequate levels in the body. However, some bacteria found in mammalian colons can synthesize folate *de novo* which can also be absorbed by the host (Stevens & Hume, 1998).

Folate has many roles in the body including amino acid metabolism, and DNA and RNA synthesis due to its role in the *de novo* synthesis of purines and pyrimidines. Folate is also essential for maintaining the methyl donor pool of S-adenosylmethionine (SAM) (Institute of Medicine (US), 1998). Folate metabolism is a part of OCM because it plays a role in providing one-carbon units for other metabolic pathways.

Folate metabolism begins in the digestive tract with the hydrolysis of the glutamate residues of food folates to produce a monoglutamate form; the folate monoglutamate, including folic acid, can then be absorbed in the duodenum and jejunum. Before entering portal circulation, all folates are reduced by the enzyme dihydrofolate reductase (DHFR) first to dihydrofolate, then to tetrahydrofolate (THF) and are further metabolized to 5-methyl-THF, the major circulating form of folate (Bailey & Caudill, 2012). After being taken up by tissues from circulation, folate must be polyglutamated so the cell is able to retain it. Folate is mainly taken up by tissues as 5-methyl-THF, though this form is a poor substrate for folylpolylglutamate synthase (FPGS) (Cichowicz & Shane, 1987). Therefore, it is thought that 5-methyl-THF conversion to THF by methionine synthase (MS) is the intracellular metabolic step that proceeds polyglutamation (Cook et al., 1987).; THF is formed after the donation of the methyl group from 5-methyl-THF and can participate in further enzymatic processes through its conversion to a variety of

compounds. These compounds participate in many pathways, each that have different metabolic roles, such as thymidylate and purine synthesis, or methionine regeneration. To reform 5-methyl-THF, THF is first converted to N_5N_{10}-methylene-THF by serine hydroxymethyltransfersae (SHMT), then to 5-methyl-THF by methylene tetrahydrofolate reductase (MTHFR).

Folate, as 5-methyl-THF, is a coenzyme for MS in the methionine metabolism pathway and donates a methyl group to homocysteine to form methionine. Methionine is activated with ATP to form SAM using methionine adenosyltransferase (MAT). SAM can be consumed by a variety of methyltransferases in numerous metabolic pathways, one of which is iAs methylation catalyzed by AS3MT. Once SAM donates its methyl group it forms S-adenosylhomocysteine (SAH); SAH is converted to homocysteine by adenosylhomocysteinase (AHCY) and the methionine metabolism cycle can continue using MS to reform methionine. Folate is important in As metabolism due to its role in the regeneration of the universal methyl donor SAM which is required for the methylation of iAs metabolites. See **Figure 3** for a scheme summarizing folate and methionine metabolism in the regeneration of SAM.

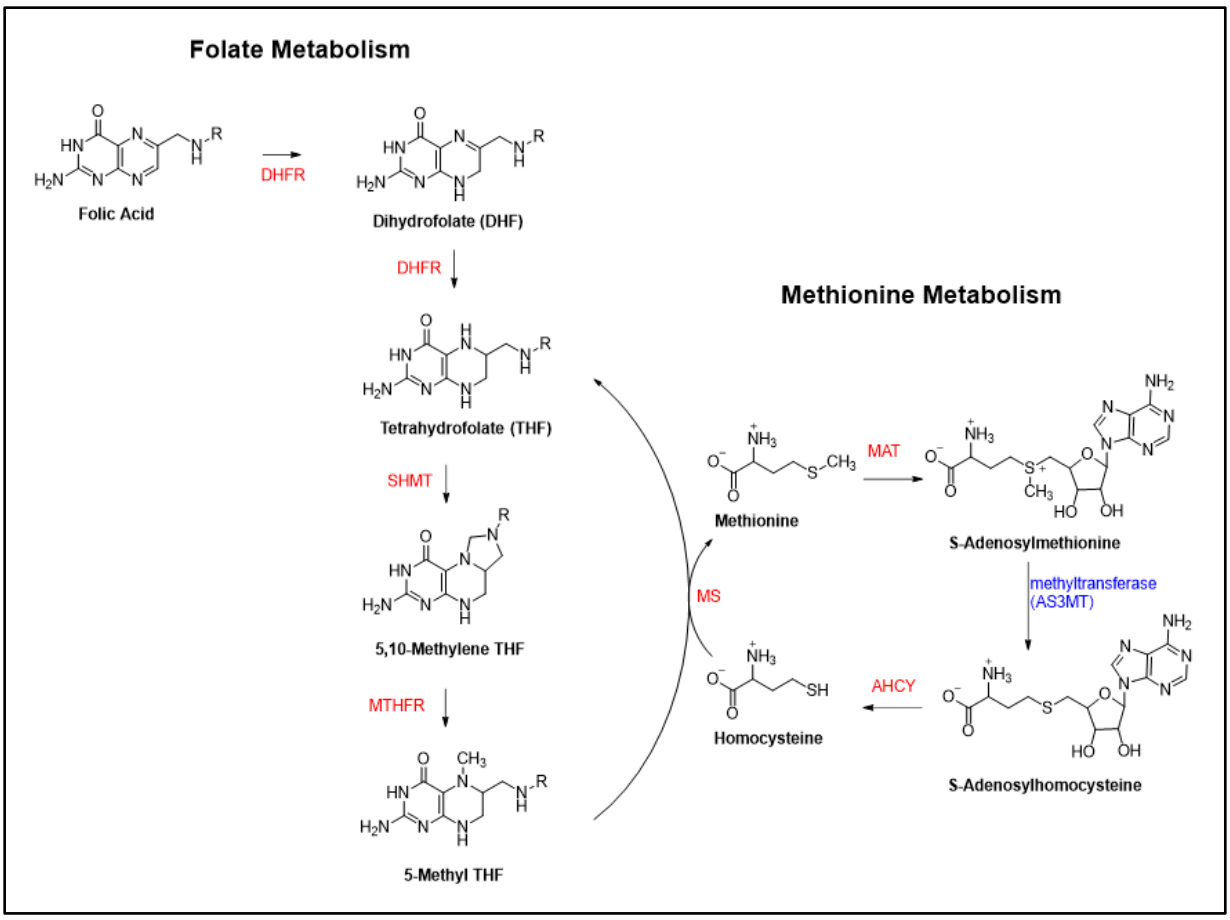

Figure 3: Folate and methionine metabolism and the regeneration of S-adenosylmethionine

Folate and Arsenic Metabolism in Epidemiological Studies

Research on the association between folate and As metabolism in human populations utilize two common methods to quantify folate status: estimation of folate intake from food dietary assessment methods or direct measurement of folate in blood and plasma. In general, studies assessing folate intake, as measured though tools such as food frequency questionnaires, have inconsistent findings relating folate intake with As metabolism. However, studies assessing folate status by measurements of plasma, serum, or erythrocyte folate levels typically find a significant association between folate status and the efficiency of iAs methylation. These studies find that lower folate levels are associated with decreased efficiency of iAs metabolism and an

increase in iAs-induced adverse health outcomes while higher folate levels and folate supplementation are associated with stimulation of iAs metabolism (Sijko & Kozłowska, 2021).

In population studies, the efficiency of iAs methylation is typically assessed using proportions of As metabolites in urine, i.e. percentage of total urinary As represented by iAs and its methylated metabolites. A review by Sijko and Kozlowska found that folate intake in adults is not associated with urinary %MAs or %DMAs in populations in many countries (Sijko & Kozłowska, 2021). However, one study has shown folate intake to be negatively associated with urinary %iAs and positively associated with the ratio of DMA/iAs in urine in a population of Mexican women, indicating that folate may stimulate iAs metabolism (López-Carrillo et al., 2016). In American Indians, high intake of B-vitamins was associated with stimulation of iAs metabolism, however the individual role of folate intake on As metabolism was not clear (Spratlen et al., 2017). Spratlen and colleagues reflect that dietary intake measures typically lead to less consistent results than studies that assess folate status through plasma folate levels (Spratlen et al., 2017). The unclear and inconsistent relationship between estimated folate intake and iAs metabolism may be due to bias associated with using dietary intake measures to estimate nutrient availability.

Unlike the dietary intake estimates, folate status, measured as plasma or serum folate concentrations, has widely been associated with efficiency of iAs metabolism in humans. However, as reviewed by Sijko and Kozlowska, the total As concentration and proportions of methylated arsenicals in urine vary between human studies (Sijko & Kozłowska, 2021). These differences are likely due to different folate intakes and arsenic exposure levels between the various study populations, though a consensus across most studies is that higher folate status is associated with stimulation of iAs metabolism. Folate deficiency has been associated with lower

capacity to methylate iAs and decreased excretion of total As in urine. Specifically, low serum and plasma folate levels are associated with increased urinary %iAs and %MAs and decreased %DMAs (Basu et al., 2011; Niedzwiecki et al., 2014). Further, increased folate levels has been associated with increased urinary %DMAs and decreased %iAs, and %MAs in blood and urine (Gamble et al., 2005; Howe et al., 2014). These studies indicate that folate plays a role in stimulating iAs methylation and excretion. Folate has also been found to play a role in decreasing As toxicity, where folate deficiency and hyperhomocysteinemia, both associated with decreased iAs methylation, are risk factors for As-induced skin lesions in Bangladeshi adults (Pilsner et al., 2009).

Studies conducted in U.S. populations where individuals have overall adequate nutrition and low levels of As exposure provide additional insight on the role of folate in iAs metabolism. One study conducted on five cycles of NHANES data (2003-2012) from the United States indicates that folate status has variable effects on iAs metabolism depending on the age of individuals. Specifically, serum folate concentration has been positively associated with urinary %MMAs in individuals < 18 years old while serum folate is positively associated with urinary %DMAs in individuals > 18 years old (Zhang et al., 2019). Another study conducted on the 2003-2004 NHANES data found that folate intake was positively associated with urinary %DMAs in adults and showed similar trends in children younger than 18 years old in crude models. In fully adjusted models, erythrocyte folate was negatively associated with urinary %iAs (Kurzius-Spencer et al., 2017). Together these results indicate that folate may play a role in iAs metabolism in populations that have overall adequate nutrition and low levels of iAs exposure (Kurzius-Spencer et al., 2017; Zhang et al., 2019).

There have been two randomized controlled trials (RCTs) investigating the association of folate supplementation with iAs metabolism and toxicity, from which numerous papers have been published. A review of most of the papers published from these two RCTs indicates that folic acid supplementation stimulates iAs metabolism (Sijko & Kozłowska, 2021). Specifically, supplementation with folic acid resulted in increased urinary %DMAs with reduced urinary %MMAs (Bozack et al., 2019, 2021; Gamble et al., 2006) and reduced total blood As levels (Gamble et al., 2007; Peters et al., 2015). Further illustrating the importance of adequate daily folate intake, Bozack and colleagues found that in groups taking folic acid supplements for 12 weeks, the cessation of folic acid supplementation for an additional 12 weeks nullified the improvement in iAs metabolism associated with prior folate supplementation (Bozack et al.2019). It was also found that supplementation with several antioxidant nutrients, including folate, may reduce the risk of As-associated skin lesions (Zablotska et al., 2008).

Folate and Arsenic Metabolism in Laboratory Studies

Research conducted in laboratory settings using mouse models have been used to further understand the role of folate in iAs metabolism, though results have been less conclusive than what has been found in human studies. To study the effects of SAM availability on iAs metabolism, a majority of mouse studies modify the intake of several OCM nutrients such as methionine, choline, and vitamin B12 in addition to folate. Few studies have assessed the specific role of folate in iAs metabolism efficiency while many more studies assess the effects of folate intake on iAs toxicity outcomes. Of the studies assessing the isolated role of modified folate intake on iAs metabolism, folate has been found to stimulate iAs metabolism to a different extent based on the length of time mice consumed the modified folate diet and the method of iAs exposure (chronic exposure vs single dose). In mice fed a folate deficient diet for 3 weeks and

given a single intraperitoneal injection of arsenate, folate deficiency was associated with lower total urinary As in male mice, though had no effect on the proportions of arsenical species (Spiegelstein et al., 2003, 2005). In contrast, high folate intake has been found to stimulate iAs metabolism and excretion of methylated species in C57BL/6J female, but not male, mice exposed to iAs for 6 weeks and 24 weeks (Huang, Douillet, Dover, Zhang, et al., 2018).

Laboratory research on the role of folate in the development of adverse iAs-associated outcomes indicates that low folate can exacerbate iAs toxicity through a variety of mechanisms while high folate has been shown to have both protective and synergistic effects on various iAs toxicity outcomes. For example, folate deficiency promotes the prevalence of arsenic-induced micronuclei (McDorman et al., 2002). In mice fed a high fat diet and exposed to iAs, low folate intake increased insulin resistance while high folate intake was protective against insulin resistance (Huang, Douillet, Dover, Zhang, et al., 2018). Folate supplementation in mice has been found to protect against many iAs-associated adverse health effects, such as iAs-induced DNA damage and hepatic and renal tissue degeneration as well as oxidative damage in cardiac tissue, with increased benefits when in combination with B12 supplementation (Acharyya et al., 2015; Bhattacharjee et al., 2013). However, high folate has also been shown to exacerbate iAs toxicity. For instance, when combined with iAs exposure *in utero*, high folate had adverse effects on DNA methylation profiles, often leading to hypermethylation of genes, though there was also some evidence of hypomethylation of genes, and acted synergistically with iAs in reducing fetal weight (Tsang et al., 2012).

Humanized Mouse Model for Arsenic Metabolism

Though mouse models are often used to study iAs metabolism and toxicity, it has long been known that iAs metabolism in mice is more efficient than in humans, resulting in faster

clearance of toxic arsenicals. To illustrate, the half-life of iAs in humans is about 4 days compared to about 2 days in mice (Vahter, 1999). In addition, the differences in iAs metabolism efficiency are also indicated by the distribution of iAs and its methylated metabolites in the urine: human urine contains 60-70% DMAs, 10-30% iAs, and 10-20% MAs, compared to the mouse urinary profile of 96-99% DMAs with trace iAs and MAs (Stýblo et al., 2019; Vahter, 1999). This is an important difference because iAs methylation efficiency correlates positively with the rate of clearance of arsenicals and negatively with their retention in tissues. For example, *As3mt* knockout mice given an oral dose of arsenate exhibit reduced iAs methylation and retain iAs in tissues to a greater extent than mice expressing *As3mt* (Drobna et al., 2009). The retention of As in both non-methylated and methylated forms enhances its toxic effects. Therefore, the difference in iAs metabolism between mice and humans raises concerns about the translatability of laboratory research when assessing the adverse effects of iAs exposure and the role of iAs metabolism as a key factor that determines these effects.

To address this concern, Koller and colleagues (Genetics Department of the UNC School of Medicine) developed a novel mouse strain humanizing the *Borcs7/As3mt* locus by syntenic replacement in 129S6/SvEvTac mice (Koller et al., 2020). These humanized AS3MT (Hu) mice have urinary As profiles representative of human urinary at both acute and subchronic iAs exposure (Koller et al., 2020). Compared to wildtype (WT) mice, Hu mice excrete less total urinary As with higher %iAs and %MAs, and lower %DMAs. After subchronic exposure to iAs in drinking water (400 μg As/L for 4 weeks), the arsenical distribution pattern in Hu mice was 48-55% DMAs, 35-47% iAs, and 5-11% MAs which compares well to human arsenical distribution of 60-80% DMAs, 10-30% iAs, and 10-20% MAs (Vahter, 1999). The retention of As was assessed primarily through quantifying arsenic metabolites in liver and kidneys and

indicated that Hu mice retain much higher levels of total As in tissues compared to WT mice. In addition, the tissue As profiles in Hu mice revealed lower efficiency to methylate iAs, as the predominant arsenical in both tissues was iAs, followed by MAs and only traces of DMAs; this is in comparison to the As profiles of WT mice where DMAs was the predominant metabolite, with low proportions of both iAs and MAs. To further validate the Hu mouse model, the amounts of total As in the liver and kidneys of these mice were compared to that predicted for tissues of humans chronically exposed to 400 μg As/L (ppb) using a human physiologically based pharmacokinetic model (El-Masri & Kenyon, 2008). The total As levels in tissues of Hu mice were much more representative of human arsenic levels than those found in tissues of WT mice, of which the values were an order of magnitude lower than the human tissue estimations.

Thus, the Hu-129S6 mice have been shown to be much better models for human iAs metabolism than the WT mice. In general, 129S6 substrains are not typically used for metabolic studies, but rather for generating genetically modified animals (*129S6*, n.d.). The strain most commonly used in biomedical research is the C57BL/6 mouse, of which there are several substrains, due to the large number of mutant mouse strains developed from this background (Birling et al., 2021). Metabolic studies and studies assessing the mechanisms of iAs metabolism in the Styblo lab and elsewhere commonly used C57BL/6 mice. One common substrain is C57BL/6J which is characterized with high susceptibility to diet-induced obesity and type 2 diabetes phenotypes such as high insulin resistance and impaired glucose tolerance (*000664 - B6 Strain Details*, n.d.). The C57BL/6J substrain has a mutated *Nnt* gene that has been associated with impaired glucose homeostasis (Freeman et al., 2006). However, another common substrain, C57BL/6N, does not have this mutation and expresses the normal *Nnt* gene (Mekada et al., 2009). Research has indicated that C57BL/6N mice have different metabolic phenotypes than

C57BL/6J mice, such as better glucose tolerance and insulin secretion with diet-induced obesity (Fontaine & Davis, 2016). Due to these genetic and phenotypic differences, it is important for researchers to make comparisons between mice of the same substrain to understand mechanistic pathways. An *As3mt*-knockout (KO) mouse model has been developed on the C57BL/6N background, first described by Drobna and colleagues (Drobna et al., 2009). This *As3mt*-KO model has been utilized to study the role of AS3MT in As metabolism and excretion (Drobna 2009; Chen 2011; Hughes 2011) as well as in the role of As retention in development of adverse metabolic phenotypes (Douillet et al., 2017; Huang et al., 2017; Huang, Douillet, Dover, Zhang, et al., 2018). To provide a more optimized model for the assessment of iAs metabolism and its metabolic effects in laboratory settings, Dr. Pardo Manuel de Villena's lab in the Genetics Department of the UNC School of Medicine developed a Hu mouse model 95% congenic with the C57BL/6N strain. **The goal of the current study was to characterize iAs metabolism in the Hu-C57BL/6N mice and to assess the role of dietary folate intake in iAs metabolism in this mouse model**.

HYPOTHESES & OBJECTIVES

We hypothesize that (a) iAs metabolism in Hu C57BL/6N mice will be less efficient than in the WT C57BL/6N mice and that (b) folate intake will affect iAs metabolism in both WT and Hu mice, with more pronounced effects in the Hu mice. The following aims will address these hypotheses:

Aim 1: Compare metabolism of a subchronic dose of iAs in WT and Hu mice. We have shown that Hu mice on 129S6 background metabolize iAs less efficiently than WT 129S6 mice. Here, we will compare iAs metabolism in male and female Hu and WT mice on C57BL/6N background. Mice will be fed a folate-adequate diet (2 mg folate/kg diet) and exposed to 0 or 400

ppb iAs in drinking water for 5 weeks. The concentrations and proportions of iAs, MAs and DMAs will be measured in urine and tissues after the exposure.

Aim 2: Characterize the effects of folate intake on iAs metabolism. In parallel with the assessment of iAs metabolism in folate-adequate mice in Aim 1, we will also examine iAs metabolism in Hu and WT mice fed a folate deficient (0 mg folate/kg diet) or a folate supplemented (10 mg folate/kg diet) diet for 6 weeks before exposure to iAs (0 or 400 ppb for 5 weeks). To assess folate status, plasma will be analyzed for folate levels before and after iAs exposure.

Exploratory Aim 3: Examine diabetogenic effects of iAs exposure in Hu and WT mice. Diabetic phenotypes have been reported in WT mice exposed to iAs for 8-20+ weeks. We hypothesize that the 5-week exposure to 400 ppb iAs will be sufficient to produce diabetes in folate deficient Hu mice. To test this hypothesis, we will measure body weight and fasting glucose and insulin levels in blood, and calculate indices that are used in clinical settings to characterize insulin resistance and β-cell function.

This research is expected to validate the C57BL/6N Hu mice as novel laboratory models for research of iAs exposure, including studies exploring the role of dietary methyl donors as potential modifiers of iAs metabolism and toxicity. Ultimately, these mouse models will improve the translatability of laboratory studies to human populations while also providing important clues about strategies for treatment or prevention of the adverse effects associated with iAs exposure.

CHAPTER 2: METHODS

Mice

Hu-C57BL/6N (Hu) mice were received in five batches (3-6 weeks old, with a range in DOB of about 2 weeks within a round) from our collaborator Dr. Fernando Pardo-Manuel de Villena in the Department of Genetics at the UNC School of Medicine. Wildtype (WT) male and female C57BL/6N mice (5-7 weeks old) were purchased from Charles River. Upon arrival, all mice were left to acclimatize to allow for the clearance of arsenic ingested from the standard grain-based rodent chow used in the respective breeding facilities. All WT mice had an acclimatization period of 12-14 days and all Hu mice had an acclimatization period of 20-27 days. During this period, mice were fed AIN-93G purified pelleted rodent diet (Round 1 mice: Dyets, Bethlehem, PA; Round 2-5 mice: Research Diets, New Brunswick, NJ) and drank DIW *ad libitum*. Mice were housed two to five per cage in a room with a 12-hour light/dark cycle. Water consumption per cage was measured weekly and food consumption per cage and body weights were measured every two weeks. A total of 430 mice made up the study cohort (n = 197 Hu, n = 233 WT).

Diets and Exposure

For each ground, the mice acclimatized until the age range of 7-9 weeks old (roughly 4 weeks of acclimatization for Hu mice and 2 weeks for WT mice). After the acclimatization period, the Hu and WT mice were placed in one of three diet groups: folate deficient (FD, 0 mg folate/kg diet), folate adequate (FA, 2 mg folate/ kg diet), or folate supplemented (FS, 10 mg/kg

diet). All diets were amino acid defined and identical in composition except for folate content and dye color (FD: Research Diets, A10012201B; FA: Research Diets, A07060801Y; FS: Research Diets, A21082701). Mice consumed the modified folate diets for 6 weeks. After 6 weeks, mice in each dietary group were exposed to 0 ppb iAs or 400 ppb iAs in DIW for an additional 5 weeks. Diet and exposure assignments in each of the 5 rounds were arranged so there were at least $n = 12$ mice per group at the end of the study.

Phenotyping

Urine Collection

Spot urine samples for As analysis were collected at baseline (before the start of the modified folate diets), pre iAs-exposure (after 6 weeks on the modified folate diets), and post iAs-exposure (after 11 weeks on the modified folate diets i.e., 5 weeks of arsenic exposure). Mice were scruffed over parafilm and spontaneous urine was collected using a pipette. Gentle pressure on the abdomen was applied to encourage urination. Urine samples were frozen at -80°C.

Plasma Folate

Fasted blood samples for plasma folate measurement were collected pre iAs-exposure (after 6 weeks on the modified folate diets) and again post iAs-exposure (after 11 weeks on the modified folate diets and 5 weeks of arsenic exposure). After a 6-hour fast, tail blood (~200 µL) was collected using lithium heparin Minivettes (Sardstedt AG & Co.; Nümbrecht, Germany). Plasma was isolated by centrifugation at 1,700g for 15 minutes at 4°C and stored at -80°C until analysis. Plasma folate levels were analyzed using Folate AccuBind ELISA Kits, following the manufacturer's protocol (Monobind Inc.; Lake Forest, CA). Plasma from mice consuming the

folate adequate and folate supplemented diets was diluted in Dulbecco's phosphate-buffered saline.

Blood Glucose and Plasma Insulin

Fasted blood samples for fasted blood glucose (FBG) and fasting plasma insulin (FPI) were collected at baseline (before the start of the modified folate diets), pre iAs-exposure (after 6 weeks on the modified folate diets), and post iAs-exposure (after 11 weeks on the modified folate diets and 5 weeks of arsenic exposure) as described above. FBG was measured using Onetouch Ultra Blue 2 glucometers (LifeScan, Inc; Malvern, PA) with One Touch Ultra glucose strips (LifeScan, Inc; Malvern, PA) and tail blood (100 µL at baseline; 200 µL at pre-iAs exposure and post iAs-exposure collections) was collected on lithium heparin Minivettes (Sardstedt AG & Co.; Nümbrecht, Germany). Plasma was isolated by centrifugation at 1,700xg for 15 minutes at 4°C and stored at -80°C. Insulin levels were analyzed using Ultra-Sensitive Mouse Insulin ELISA kits following the manufacturer's protocol (Crystal Chem; Elk Grove Village, IL).

Metabolic Indices

Metabolic indices including homeostatic model assessment for insulin resistance (HOMA-IR) index and HOMA of β-cell function (HOMA-B) index as follows:

$$\text{Insulin}\ \frac{\mu\text{IU}}{\text{mL}} = \text{Insulin}\ \frac{\text{ng}}{\text{mL}} \times (10^4/348)$$

$$\text{HOMA-IR} = \left[\left(\text{FBG, in}\ \frac{\text{mg}}{\text{dL}}\right) \times \left(\text{FPI, in}\ \frac{\mu\text{IU}}{\text{mL}}\right)\right]/405$$

$$\text{HOMA-B} = \left[\left(\text{FPI, in}\ \frac{\mu\text{IU}}{\text{mL}}\right) \times 20\right]/\left[\left(\text{FBG, in}\ \frac{\text{mmol}}{\text{L}}\right) - 3.5\right]$$

Sacrifice and Tissue Collection

At the end of the iAs-exposure period, the mice (at that time 19-21 weeks old) were sacrificed by cervical dislocation without anesthesia. Submandibular blood (non-fasted) and tissues were collected at sacrifice, including liver, kidneys, pancreas, spleen, heart, right lung, adrenals, bladder, white adipose (gonadal), brown adipose, calf muscle, testes or ovaries, small intestine, caecum, and colon. All tissues were flash-frozen in liquid nitrogen and stored at −80°C until analysis.

Arsenic Speciation Analysis

The concentrations of arsenic species (iAs, MAs and DMAs) in samples of spot urine and in 20% liver homogenates prepared in DIW were measured using hydride generation–cryotrapping with inductively coupled plasma mass spectrometry (HG-CT-ICP-MS) as previously described (Matousek, 2013). Samples were treated with 2% L-cysteine (Sigma-Aldrich; St. Louis, MO) at room temperature for 1 hour to reduce pentavalent iAs species to their trivalent counterparts (Matoušek et al., 2008). The concentration of total arsenic was calculated as the sum of arsenic species represented by iAs (iAs^{III} + iAs^{V}), MAs (MAs^{III} + MAs^{V}), and DMAs ($DMAs^{III}$ + $DMAs^{V}$).

Statistical Analysis

All data was assessed for outliers using Grubbs test for outliers. For bodyweights, data was analyzed by ANOVA with Student's post-hoc *t*-tests using JMP Pro 16.1.0 (SAS Institute, Cary, NC). After exclusion of the outliers, all remaining data was analyzed using Kruskal-Wallis test with Dunn's multiple comparisons post-test or Welch unequal variance *t*-test using

GraphPad InStat v3.01 (La Jolla, CA). The p-value of 0.05 was determined *a priori* to be the cut off for statistical significance.

CHAPTER 3: RESULTS

Survival of Mice in the Study

Over the course of the study, 5% of the mice were removed because of aggression between co-housed male mice or due to natural causes. This includes 13 WT males (1 control, unexposed mouse on the FD diet; of those on the FA diet, 1 was exposed to iAs and 2 were control; and of those on the FS diet, 3 were exposed to iAs and 6 were control), 7 Hu males (of those on the FD diet, 4 were exposed to iAs and 1 was control; 2 controls on the FS diet), and 2 Hu females on the FD diet unexposed to iAs.

Food and Water Consumption

Food consumption per cage was measured biweekly (**Figure 4**). Males typically consumed between 2 and 4 g food/day per mouse while females typically consumed between 1.5 and 3.5 g food/day per mouse. There were no significant differences in daily food consumption per mouse among males or females between diet groups or iAs exposures. However, starting at 9 weeks old, Hu mice tended to consume more food per day than WT mice.

Water consumption per cage was measured weekly (**Figure 5**). Hu mice typically consumed between 2 and 6 g water/day while WT mice typically consumed between 2 and 4 g water/day per mouse. Among both males and females, Hu mice tended to consume more water than WT mice regardless of diet or iAs exposure.

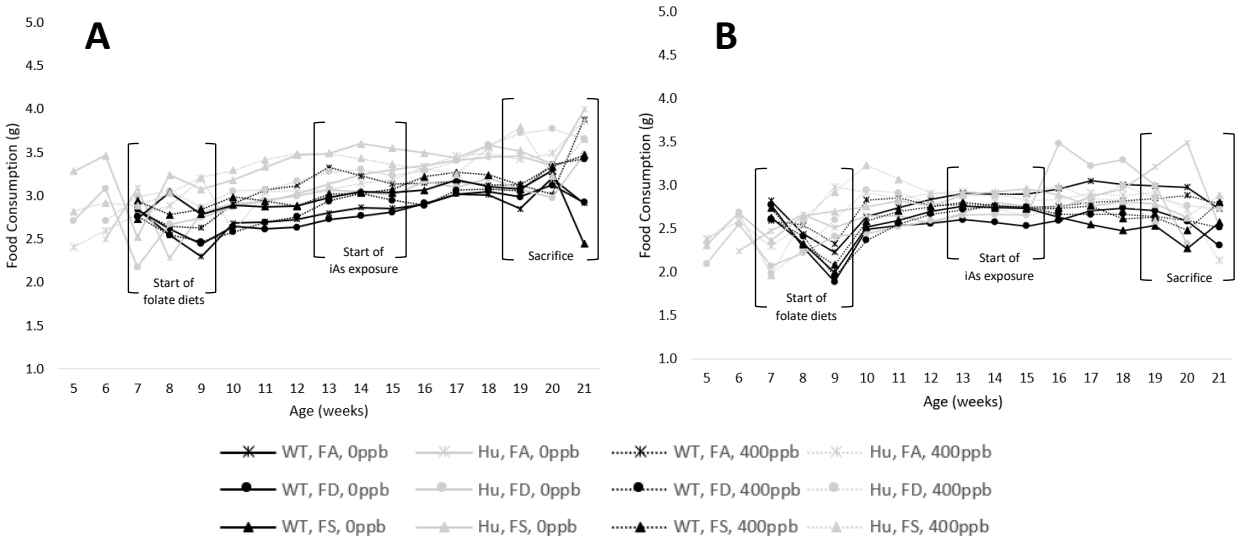

Figure 4: Average daily food consumption per mouse among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0 or 400 ppb iAs) for **(A)** males and **(B)** females. N = 12-29 per group.

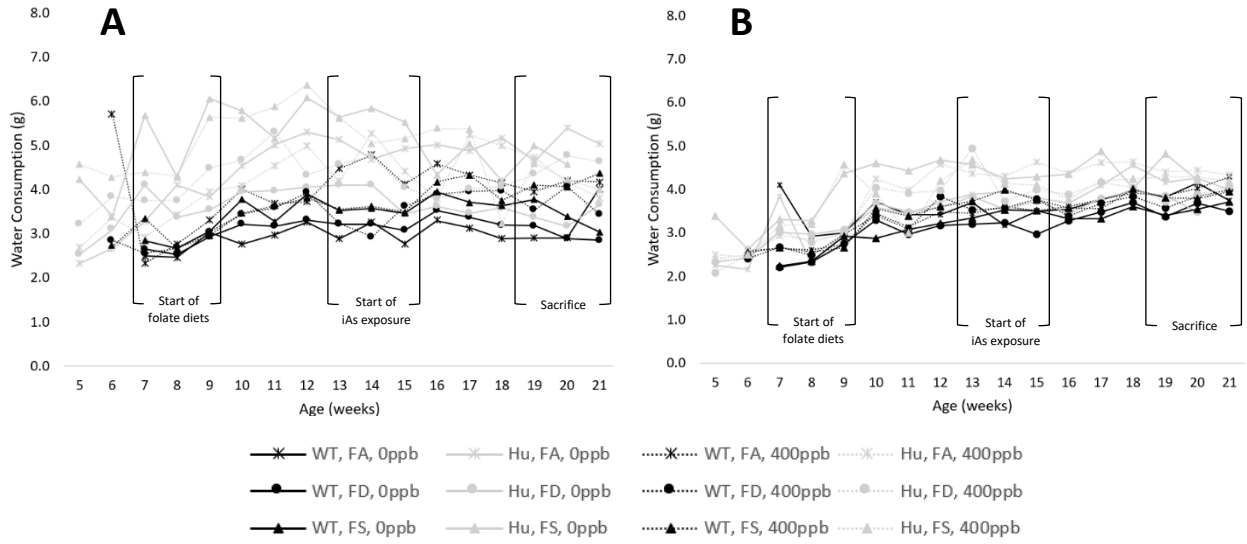

Figure 5: Average daily water consumption per mouse for **(A)** males and **(B)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0 or 400 ppb iAs). N = 12-29 per group.

Body Weights

Body weights of all mice were measured biweekly (**Figure 6**). At all timepoints, males were heavier than their female counterparts. Hu males had significantly higher body weight than WT males at all time points. Interestingly, WT females tended to have higher body weights than Hu females until 11 weeks old. However, Hu females began to weigh more than WT females at 12 weeks old. The differences in body weight among females were statistically significant only at 7, 16, and 19 weeks of age.

Mice began consuming the modified folate diets (folate deficient, FD; folate adequate, FA; folate supplemented, FS) at 7-9 weeks old. Among Hu males, those consuming the FS diet had higher body weights than those consuming the FA or FD diet starting at 9 weeks old ($p < 0.05$). These differences were not observed among WT males. Within each diet group, Hu males had higher body weights than their WT counterparts from 8-13 weeks old ($p < 0.05$), with the exception of at 11 weeks old where differences were not significant in the FA diet. In general, there were no statistically significant differences between Hu and WT females in the first 6 weeks of consuming the modified folate diets. In females, there were generally no significant effects of folate intake on body weight. WT females consuming the FS diet had consistently lower body weights than those consuming the FA diet, though this was only significant at 10 and 11 weeks old ($p < 0.05$).

Starting at 13-15 weeks old, mice were exposed to either 0 ppb or 400 ppb iAs in drinking water while continuing to consume the modified folate diets. There were no significant differences in body weights due to iAs exposure among male or female mice regardless of diet or genotype. Folate intake continued to have no significant effects on body weight among WT males. Folate supplementation in Hu males continued to result in higher body weights compared

to Hu males consuming the FA and FD diet, though iAs exposure mitigated the differences so they were not always significant. Among females, there were no significant differences in body weight due to genotype or folate intake through the iAs exposure period.

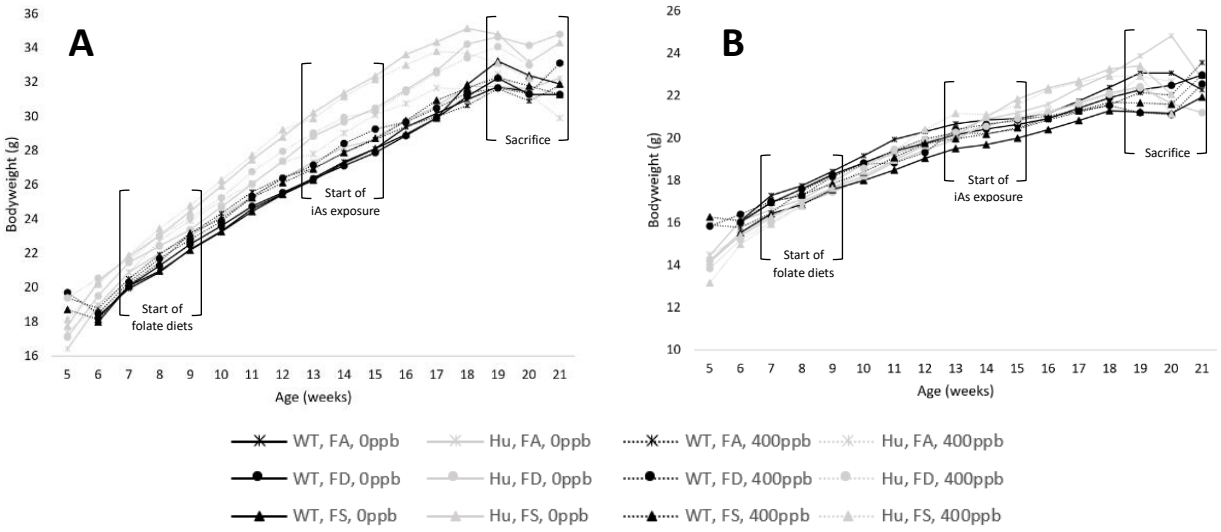

Figure 6: Average body weights of mice among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0 or 400 ppb iAs) for **(A)** males and **(B)** females. N = 12-29 per group.

Plasma Folate

Plasma folate was measured after 6 weeks on the modified folate diets (i.e. pre iAs-exposure timepoint) and again after 5 weeks of exposure with continuing consumption of the modified folate diets (i.e, post iAs-exposure timepoint after 11 weeks total consuming the folate diets). As expected, there were statistically significant differences in plasma folate levels of mice consuming low, adequate, and high folate at both 6 weeks (**Table 1**) and 11 weeks (**Table 2**) of consuming the modified folate diets.

Pre iAs-Exposure Plasma Folate

Prior to iAs exposure, after 6 weeks of consuming the modified folate diets, mice on the FD diet had lower fasted plasma folate than those consuming the FA and FS diets regardless of genotype or sex ($p < 0.05$) (**Table 1**). Taking the average of all groups, the plasma folate in mice consuming the FD diet was 9-fold lower than those consuming the FA diet. Mice consuming the FS diet had significantly higher fasted plasma folate than those consuming the FA diet for all groups, with the exception of WT females where differences did not reach statistical significance ($p < 0.05$). Taking the average of all groups, mice consuming the FS diet had a 53% increase in plasma folate compared to the FA diet.

While there were no significant differences in plasma folate levels due to genotype, Hu mice tended to have higher plasma folate levels when consuming the FD and FS diet, though lower plasma folate when consuming the FA diet, compared to WT mice. There were notable sex-related differences where the FD diet resulted in lower fasted plasma folate in males as compared to their female counterparts for both genotypes ($p < 0.05$). In contrast, the FS diet resulted in higher plasma folate in males compared to their female counterparts for both genotypes ($p < 0.05$). There were no significant sex-related differences in the FA diet for either genotype.

Table 1: Pre iAs-exposure folate levels in plasma of male and female WT and Hu mice after consuming folate deficient, folate adequate, or folate supplemented diets for 6 weeks. (ng/mL; Mean ± SD)

	Male		Female	
Diet	WT	Hu	WT	Hu
Folate Deficient	12.9 ± 5.7 [a,c,d]	13.1 ± 4.0 [a,c,d]	18.6 ± 4.0 [a,c,d]	22.7 ± 5.0 [a,c,d]
Folate Adequate	154.6 ± 45.1 [c,e]	142.1 ± 37.0 [c,e]	142.1 ± 31.4 [c]	137.9 ± 26.9 [c,e]
Folate Supplemented	239.1 ± 39.0 [a,d,e]	255.2 ± 29.6 [a,d,e]	172.0 ± 25.0 [a,d]	188.8 ± 25.2 [a,d,e]

Note: Data are shown for n = 12-50 mice per group.
[a] *P<0.05* comparing males vs. females of the same genotype and diet, using Welch's t-test for unequal variance
[b] *P<0.05* comparing WT vs. Hu of the same sex and diet, using Kruskal-Wallis test
[c] *P<0.05* comparing FD vs. FA of the same sex and genotype, using Kruskal-Wallis test
[d] *P<0.05* comparing FD vs. FS of the same sex and genotype, using Kruskal-Wallis test
[e] *P<0.05* comparing FA vs. FS of the same sex and genotype, using Kruskal-Wallis test

Post iAs-Exposure Plasma Folate

Plasma folate was measured for a second time after the iAs exposure period, after 11 weeks of consuming the modified folate diets. Among all mice, modified folate intake resulted in differences in plasma folate consistent with that seen after 6 weeks of consuming the folate diets, though differences were not always statistically significant (**Table 2**). Taking an average of all groups, mice consuming the FD diet had a 10-fold reduction in plasma folate compared to the FA diet. Among males, folate deficiency resulted in significantly lower plasma folate compared to those consuming the FA diet for both genotypes, regardless of iAs exposure ($p < 0.05$). Similar trends were found among females, though the difference was not significant when combined with iAs exposure in WT females. Taking an average of all groups, mice on the FS diet had a 70% increase in plasma folate compared to mice on the FA diet. However, the increase in plasma folate in the FS diet was not always significant compared to the FA diet among males and was not significant among females, regardless of genotype or iAs exposure.

Exposure to iAs resulted in lower plasma folate levels among Hu males on FA diet but higher plasma folate in Hu males on the FS diet compared to control, unexposed Hu males in the corresponding dietary groups. This effect of iAs exposure was not observed in WT males, though iAs exposure tended to result in lower plasma folate for all diet groups compared to controls. iAs exposure also tended to result in lower plasma folate in both Hu and WT females for all diet groups, though differences were not statistically significant.

Consistent with the pre iAs-exposure timepoint, there were no statistically significant differences in plasma folate levels due to genotype at the post iAs-exposure timepoint. However, both WT and Hu females maintained higher plasma folate compared to males when consuming the FD diet regardless of iAs exposure ($p < 0.05$). Both WT and Hu males maintained higher plasma folate compared to females when consuming the FA and FS diets ($p < 0.05$), though differences were not significant among control Hu mice not exposed to iAs. Interestingly, differences due to sex became significant for mice consuming the FA diet after the iAs exposure period where male mice had higher fasted plasma folate than their female counterparts, though only in the non-exposed groups ($p < 0.05$).

Table 2: Post iAs-exposure folate levels in plasma of male and female WT and Hu mice consuming folate deficient, folate adequate, or folate supplemented diets for 11 weeks with 5 weeks of exposure to 0 ppb or 400 ppb iAs in drinking water. (ng/mL; Mean ± SD)

	Male		Female	
Diet and Exposure	WT	Hu	WT	Hu
Folate Deficient				
0 ppb iAs	6.4 ± 2.6 [a,c,d]	8.3 ± 3.3 [a,c,d]	10.8 ± 3.2 [a,c,d]	16.2 ± 4.2 [a,c,d]
400 ppb iAs	5.4 ± 2.1 [a,c,d]	7.8 ± 3.2 [a,c,d]	10.9 ± 4.2 [a,d]	13.0 ± 4.4 [a,c,d]
Folate Adequate				
0 ppb iAs	105.8 ± 26.8 [a,c,e]	125.0 ± 20.0 [a,c,f]	81.9 ± 23.6 [a,c]	96.7 ± 26.0 [a.c]
400 ppb iAs	97.5 ± 21.3 [c,e]	105.3 ± 28.3 [c e,f]	74.4 ± 29.4	88.0 ± 32.9 [c]
Folate Supplemented				
0 ppb iAs	176.6 ± 17.7 [a,d,e]	175.1 ± 33.4 [d,,f]	153.3 ± 33.5 [a,d]	157.8 ± 35.6 [d]
400 ppb iAs	173.2 ± 25.1 [a,d,e]	197.7 ± 20.9 [a,d,e,f]	142.3 ± 44.8 [a,d]	130.1 ± 34.4 [a,d]

Note: Data are shown for n = 12-28 mice per group.
[a] *P<0.05* comparing males vs. females of the same genotype, diet, and iAs exposure, using Welch's t-test for unequal variance
[b] *P<0.05* comparing WT vs. Hu of the same sex, diet, and iAs exposure, using Kruskal-Wallis test
[c] *P<0.05* comparing FD vs. FA of the same sex, genotype, and iAs exposure, using Kruskal-Wallis test
[d] *P<0.05* comparing FD vs. FS of the same sex, genotype, and iAs exposure, using Kruskal-Wallis test
[e] *P<0.05* comparing FA vs. FS of the same sex, genotype, and iAs exposure, using Kruskal-Wallis test
[f] *P<0.05* comparing 0 ppb vs. 400 ppb of the same sex, genotype, and diet, using Kruskal-Wallis test

Change in Plasma Folate from Pre iAs-Exposure to Post iAs-Exposure

There was wide variation in the change in plasma folate levels among mice consuming the FA and FS diet, regardless of genotype, sex, or iAs exposure level (**Figure 7**). In contrast, differences in plasma folate among mice consuming the FD diet had relatively low variation regardless of genotype, sex, or iAs exposure. Plasma folate was lower after 11 weeks (i.e. post iAs-exposure) compared to after 6 weeks (i.e. pre iAs-exposure) of consuming the modified folate diets for all groups. Males and females consuming the FS diet had a greater reduction in plasma folate compared to the FD group for both genotypes, though differences were only significant among males. iAs exposure did not have clear effects on the change in plasma folate from 6 to 11 weeks of consuming the modified folate diets. However, iAs exposure in Hu males consuming the FS diet resulted in a significantly smaller decrease in plasma folate compared to

their non-exposed counterparts ($p < 0.05$). WT and Hu males consuming the FS diet had a significantly larger decrease in plasma folate compared to their female counterparts, though only among non-exposed mice ($p < 0.05$).

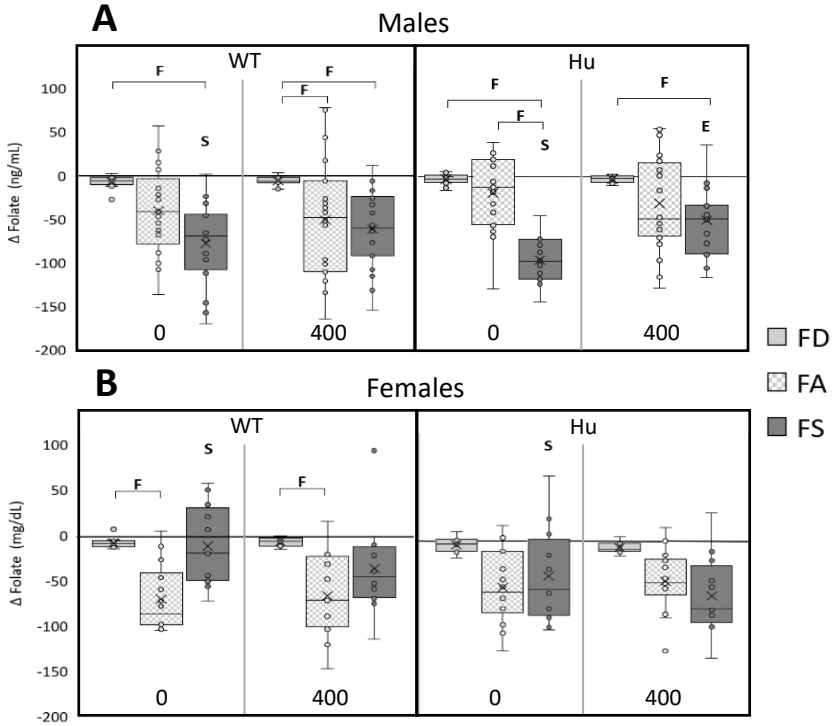

Figure 7: Change in fasted plasma folate from pre iAs-exposure (after 6 weeks of consuming the diets) to post iAs-exposure (after 11 weeks of consuming the diets). Change in fasting plasma folate in **(A)** males and **(B)** females and among groups split by genotype (WT or Hu) and folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0 ppb or 400 ppb). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 12-28). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparison: S, males vs. females of the same genotype, diet, and iAs exposure; F, comparisons of diets among the same sex, genotype, and iAs exposure; E, 0 ppb vs. 400 ppb of the same sex, genotype, and diet.

Arsenic Species in Urine

Urine was collected at baseline before the start of the modified folate diets, after 6 weeks on the modified folate diets (i.e. pre iAs-exposure), and after 5 weeks of iAs exposure. Urine from the post iAs-exposure timepoint has been analyzed for a subsection of the study cohort to

provide preliminary data on the distribution of iAs and its methylated metabolites in the urine of WT and Hu mice consuming the modified folate diets in combination with 0ppb or 400 ppb iAs exposure for 5 weeks. As expected, mice consuming 400 ppb iAs had significantly higher total arsenic (tAs) in spot urine compared to mice consuming 0 ppb iAs (**Figure 8**).

The Hu mice tended to excrete less tAs in urine compared to WT mice. However, this difference was statistically significant only among iAs-exposed males fed the FD diet and iAs-exposed females fed the FD and FA diets (**Figure 8 B**). Folate intake did not significantly affect tAs levels in urine in either genotype. The apparent differences between the FD and FS groups exposed to iAs suggest an increase in tAs excretion by FS in Hu males and females but did not reach statistical significance with the given number of replicates.

Overall, Hu mice excreted higher %iAs and %MAs with lower %DMAs compared to WT mice, though differences were not always significant (**Figure 9**). In fact, the majority of statistically significant differences in the proportions of urinary arsenicals were seen in mice exposed to 400 ppb iAs. There were no significant differences in the proportions of arsenicals in the urine of mice due to folate intake, regardless of genotype or iAs exposure.

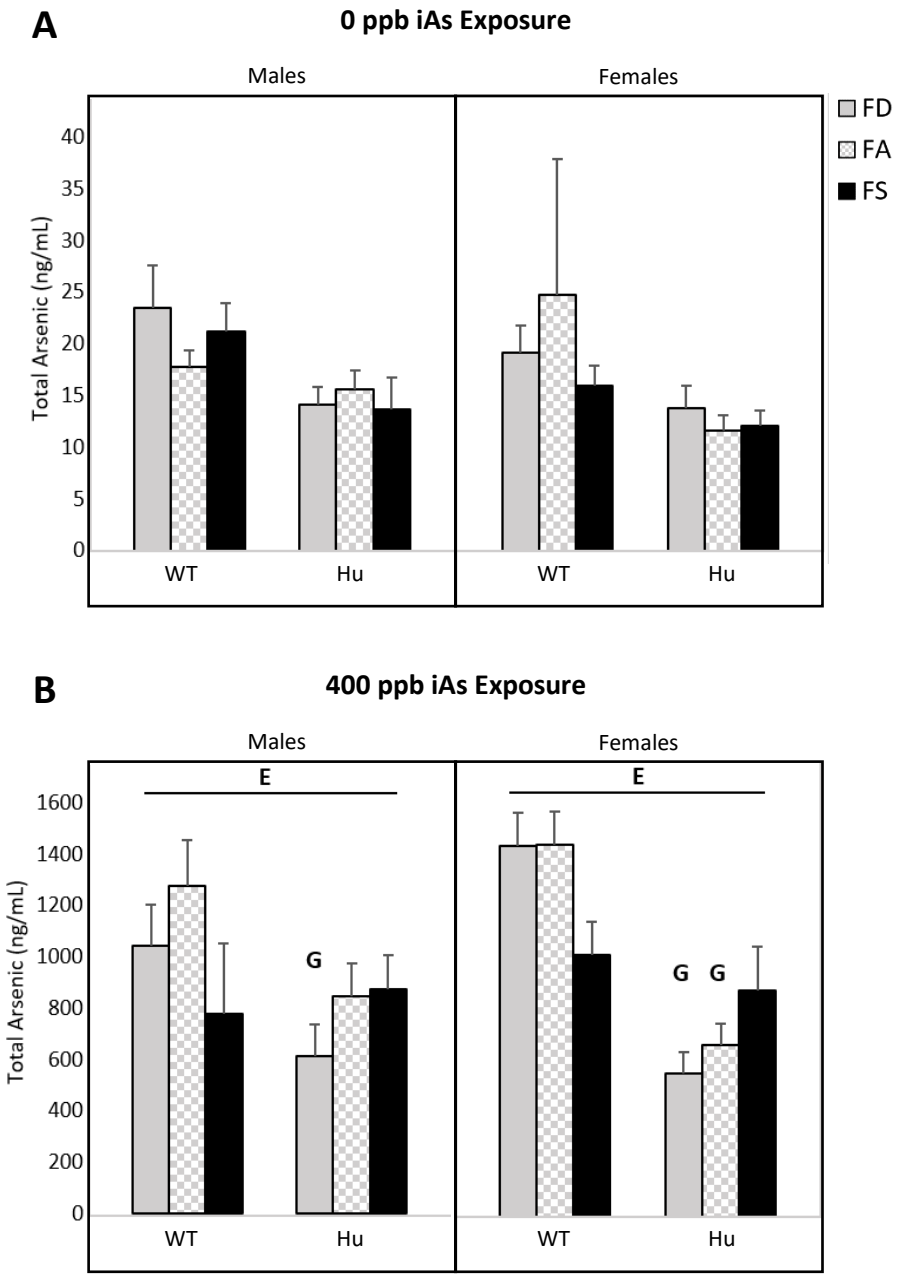

Figure 8: Post iAs-exposure total As in spot urine collected after 11 weeks of consuming the modified folate diets with 5 weeks of exposure to 0 ppb or 400 ppb iAs. Total urinary As in **(A)** males and **(B)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0 ppb or 400 ppb). Mean + SE for each group (n = 4-12; only rounds 1-3 represented). $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparison: G, WT vs. Hu of the same sex, diet, and iAs exposure; E, 0 ppb vs 400 ppb among the same sex, diet, and iAs exposure.

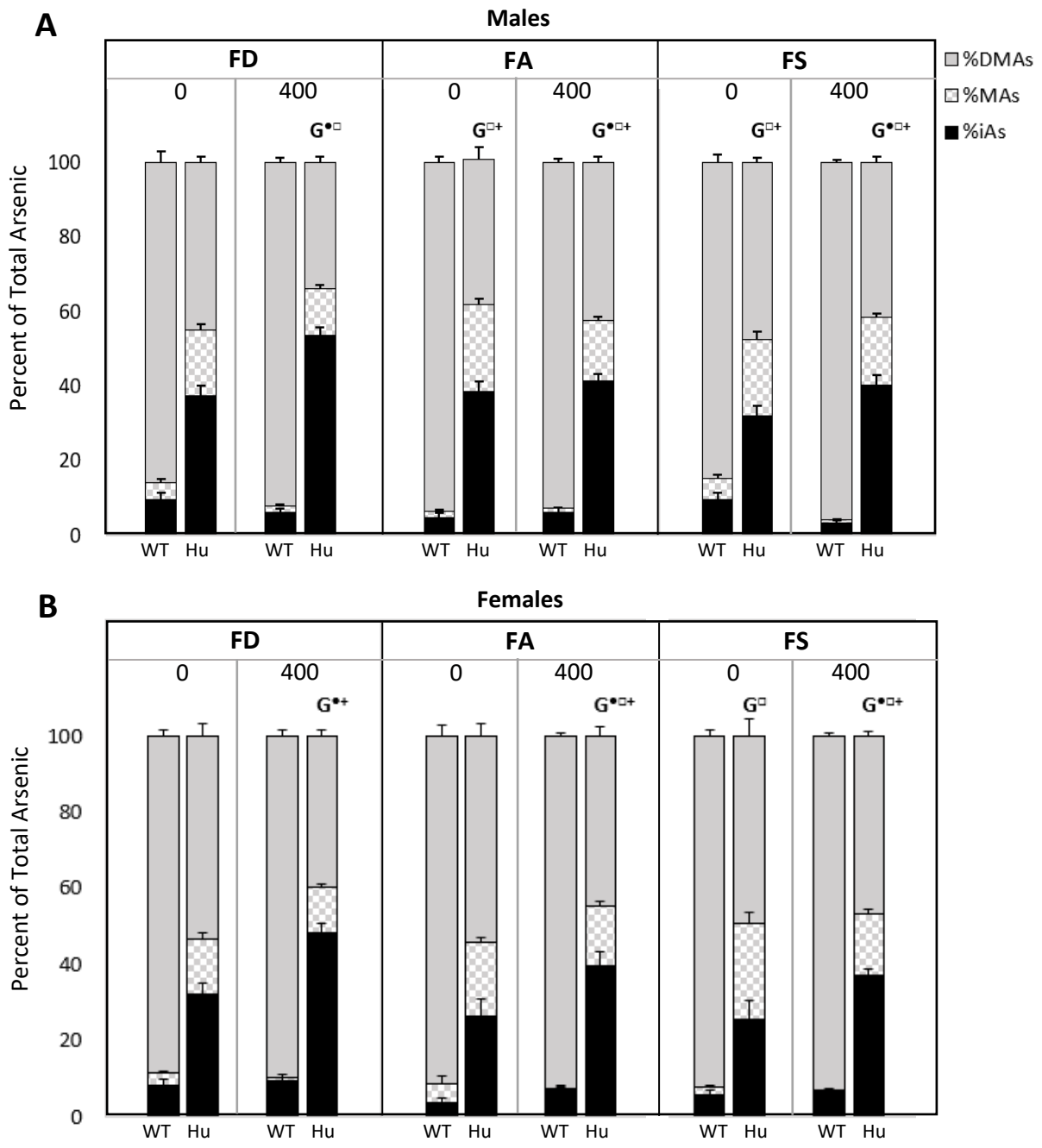

Figure 9: Post iAs-exposure urinary As profiles after 11 weeks of consuming the modified folate diets with 5 weeks of exposure to 0 ppb or 400 ppb iAs. The proportions of total As represented by inorganic arsenic (%iAs), methyl-arsenic (%MAs), and dimethyl-arsenic (%DMAs) for males and **(B)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0ppb or400 ppb). (Mean + SE) for each group (n = 4-12; only rounds 1-3 represented). $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparison: G, WT vs. Hu of the same sex, diet, and iAs exposure. Significant differences for metabolites indicated by: ● iAs, □ MAs, + DMAs

Arsenic Species in Liver

Mouse tissues were collected at sacrifice, after 11 weeks of consuming the modified diets combined with iAs exposure during the last 5 weeks. The livers have been analyzed for tAs and proportions of arsenicals for a subset of the study cohort to provide preliminary data on the iAs retention patterns in the WT and Hu mice. As expected, mice consuming 400 ppb iAs had higher tAs in livers compared to mice consuming 0 ppb iAs (**Figure 10**). The exposure-related differences in tAs were statistically significant for all groups except among WT females consuming the FD diet where differences did not reach significance ($p < 0.05$).

The Hu mice retained more tAs in the liver compared to WT mice, regardless of diet or iAs-exposure group. There appears to be a sex-dependent effect in iAs retention among Hu mice only, where females tend to retain more tAs in the liver than males, though differences did not reach statistical significance for all diets ($p < 0.05$). Folate intake did not significantly affect tAs levels in the livers of mice in either genotype. The apparent differences in Hu females between the FD and FS groups exposed to iAs suggest an increase in tAs excretion with FS but did not reach statistical significance with the given number of replicates.

Overall, Hu mice retained higher %iAs with lower %DMAs compared to WT mice, though differences were only statistically significant among iAs-exposed groups (**Figure 11**). Hu mice also tended to retain higher %MAs compared to WT mice, though differences were statistically significant among iAs-exposed mice consuming the FA and FS diets. There were sex-dependent differences in the proportion of retained arsenicals in Hu mice, where females tended to retain lower %iAs and higher %MAs compared to males; the difference in proportions of iAs and MAs were statistically significant for all groups except iAs-exposed mice consuming

the FA diet ($p < 0.05$). There were no significant differences in the proportions of arsenicals in the livers of mice due to folate intake, regardless of genotype or iAs exposure.

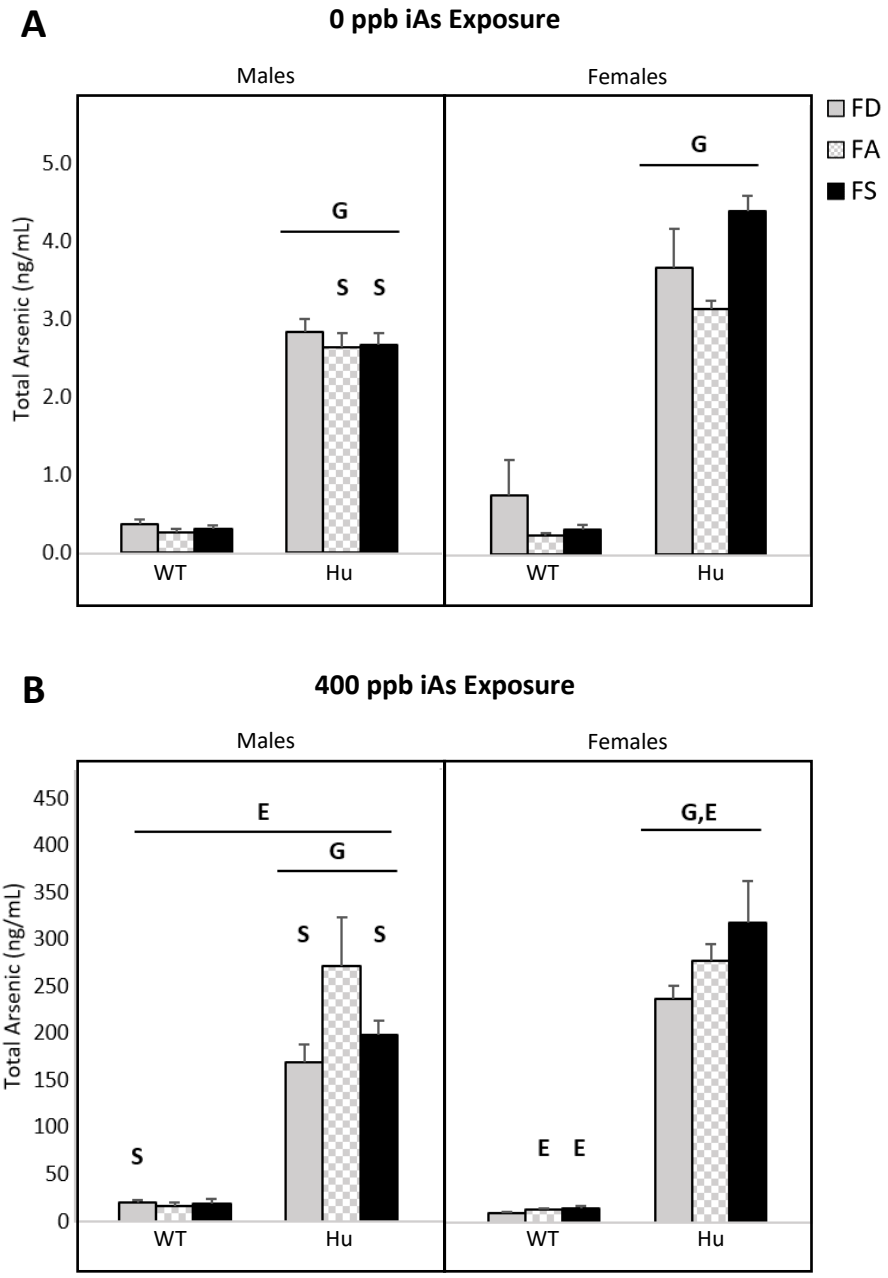

Figure 10: Total As in livers after 11 weeks of consuming the modified folate diets with 5 weeks of exposure to 0 ppb or 400 ppb iAs. Total As in 20% liver homogenate in DIW for **(A)** males and **(B)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0 ppb or 400 ppb). Mean + SE for each group (n = 6-12; only rounds 1-3 represented). $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparison: G, WT vs. Hu of the same sex, diet, and iAs exposure; S, males vs. females of the same genotype and diet; E, 0 ppb vs 400 ppb among the same sex, diet, and iAs exposure.

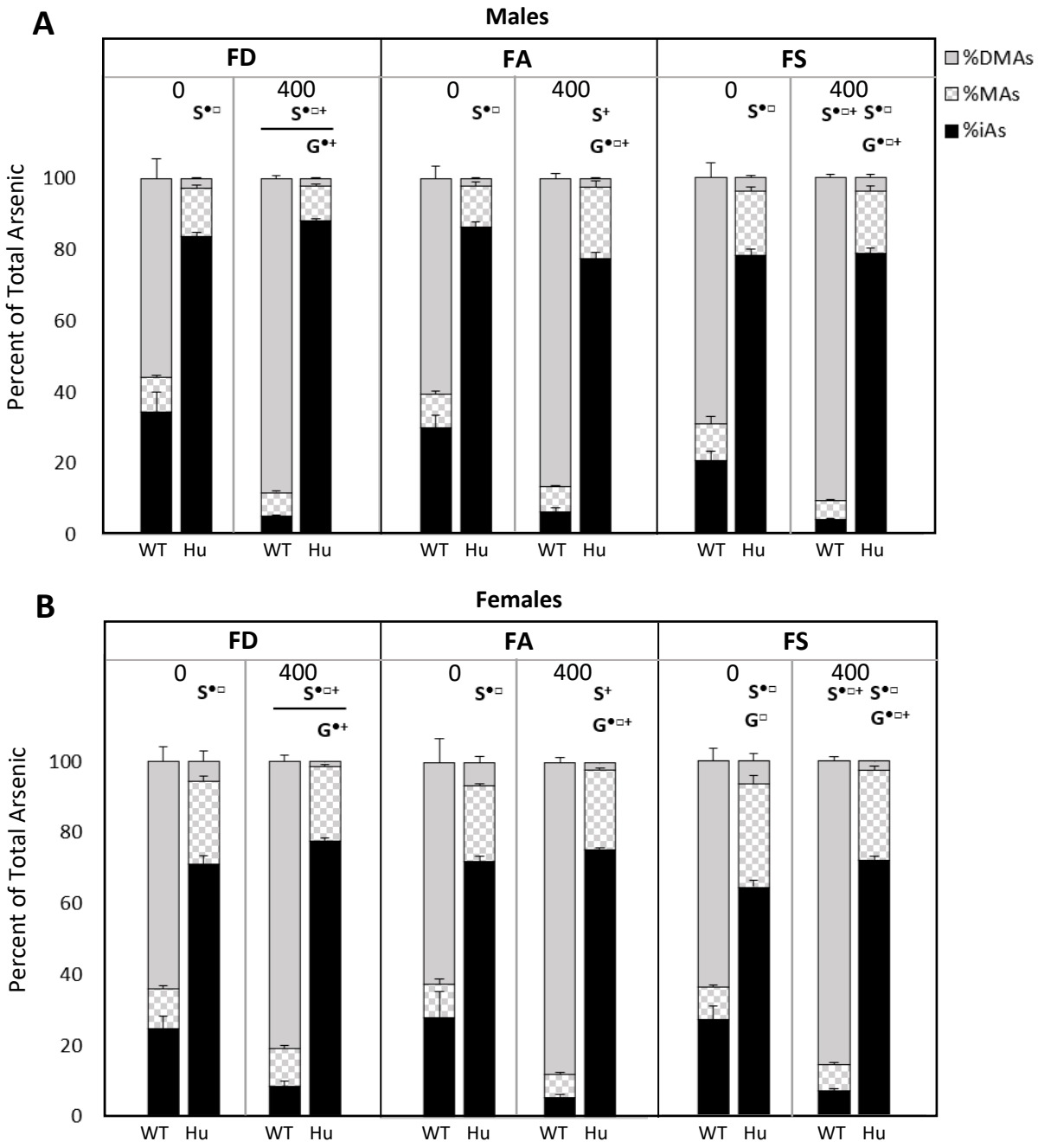

Figure 11: Liver As profiles after 11 weeks of consuming the modified folate diets with 5 weeks of exposure to 0 ppb or 400 ppb iAs. The proportions of total As represented by inorganic arsenic (%iAs), methyl-arsenic (%MAs), and dimethyl-arsenic (%DMAs) in 20% liver homogenates in DIW for males and **(B)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0ppb or400 ppb). (Mean + SE) for each group (n = 6-12; only rounds 1-3 represented). $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparison: G, WT vs. Hu of the same sex, diet, and iAs exposure; S, males vs. females of the same genotype and diet. Significant differences for metabolites indicated by: ● iAs, □ MAs, + DMAs

Diabetes Phenotypes and Metabolic Indices

Fasted blood glucose (FBG) and fasting plasma insulin (FPI) was measured at baseline before the start of the modified folate diets, after 6 weeks on the modified folate diets (i.e., pre iAs-exposure), and after 5 weeks iAs exposure. From the FBG and FPI values, homeostatic model assessment for insulin resistance (HOMA-IR) index and β-cell function (HOMA-B) were calculated at each timepoint as a proxy to estimate insulin resistance and pancreatic beta cell function, respectively.

Baseline

Males had higher FBG and FPI at baseline compared to their female counterparts regardless of genotype ($p < 0.05$) (**Figure 12**). Hu mice had higher FBG and lower FPI at baseline compared to WT mice, though differences were statistically significant only among males ($p < 0.05$). Males had significantly higher HOMA-IR and HOMA-B than females regardless of genotype ($p < 0.05$). Hu mice had lower HOMA-B than WT mice regardless of sex ($p < 0.05$). In addition, Hu mice tended to have lower HOMA-IR than WT mice, though these differences were statistically significant only among females ($p < 0.05$).

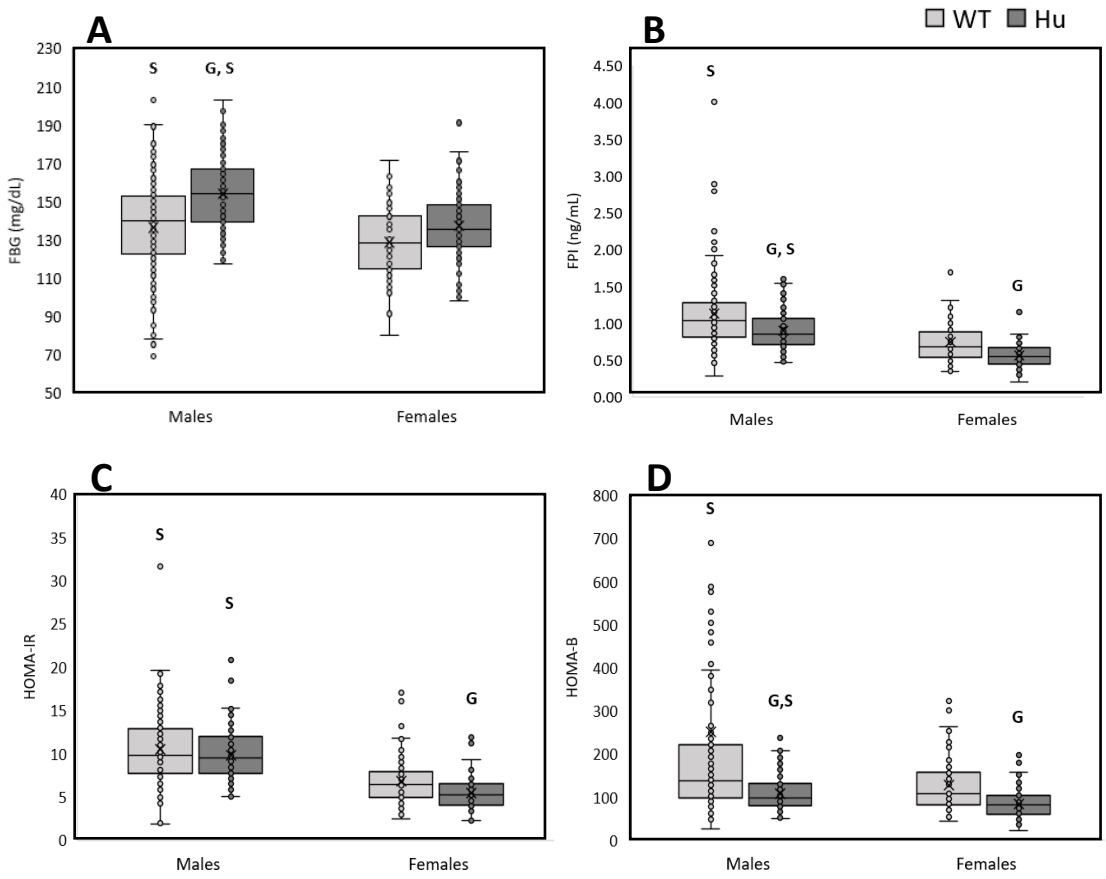

Figure 12: Baseline **(A)** Fasting blood glucose, **(B)** Fasting plasma insulin, **(C)** HOMA-IR, and **(D)** HOMA-B of mice among groups split by genotype (WT or Hu) and sex after 2 weeks of acclimatization. Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 77-153). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Kruskal-Wallis test for the following comparison: G, WT vs. Hu of the same sex; S, males vs. females of the same genotype.

Pre iAs-exposure

Prior to iAs exposure, after 6 weeks of consuming the modified folate diets, WT males had higher FBG than WT females in all diet groups ($p < 0.05$) (**Figure 13 A, B**). The same trends were seen in Hu mice, though differences were only significant for the FD diet ($p < 0.05$). Hu males consuming the FD diet had in higher FBG compared to the FA or FS diet ($p < 0.05$). Males had higher FPI than females in all diet groups, regardless of genotype ($p < 0.05$) (**Figure 13 C, D**). Folate intake did not result in any significant differences in FPI among males or

females of either genotype. There were no significant differences in FBG or FPI between WT and Hu mice.

Both WT and Hu males had higher HOMA-IR values compared to their female counterparts regardless of folate intake ($p < 0.05$) (**Figure 14 A, B**). Males tended to have higher HOMA-B values than females, though differences were not always significant (**Figure 14 C, D**). The differences in HOMA-B were significant in WT and Hu mice consuming the FD diet as well as in Hu mice consuming the FS diet ($p < 0.05$). Among Hu males, those consuming the FA diet had lower HOMA-B compared to those consuming either the FD or FS diet, though this difference was only significant compared to Hu males consuming the FD diet ($p < 0.05$).

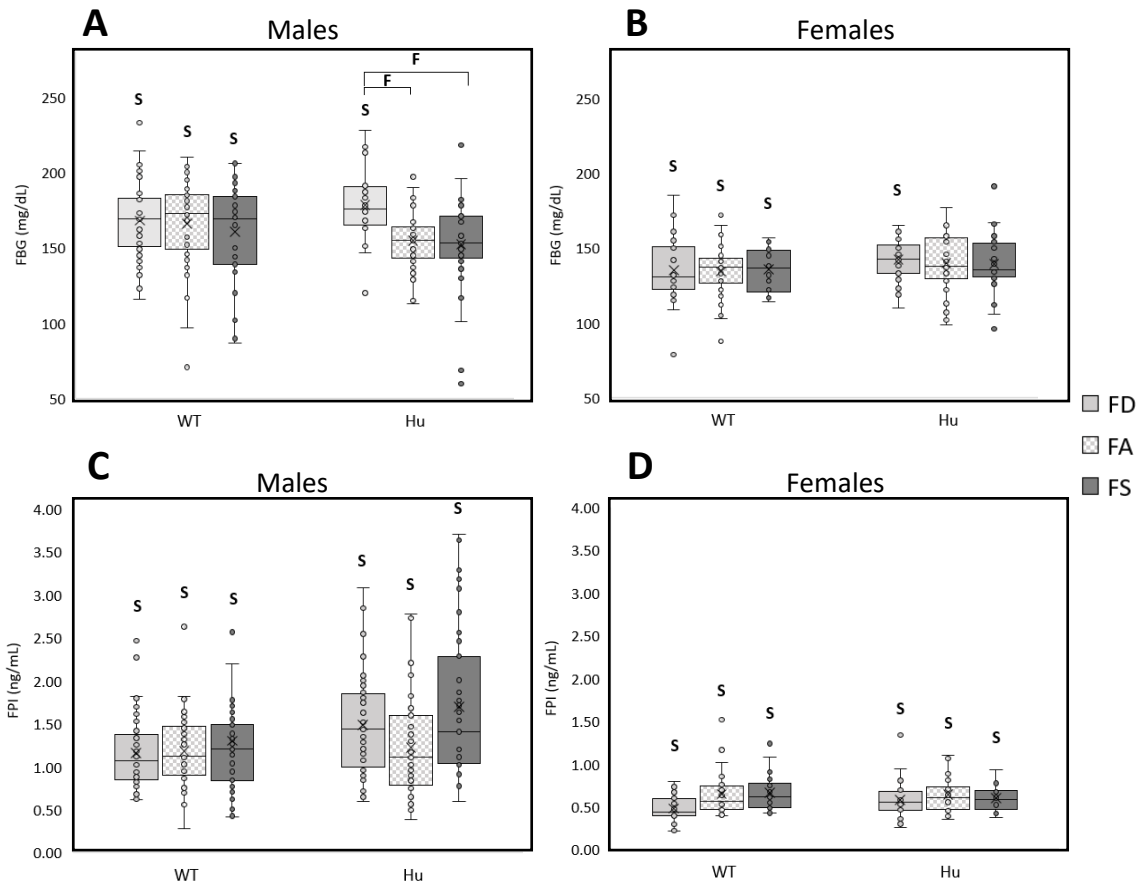

Figure 13: Pre iAs-exposure fasting blood glucose and fasting plasma insulin after 6 weeks of consuming the modified folate diets. Fasting blood glucose in **(A)** males and **(B)** females and fasting plasma insulin in **(C)** males and **(D)** females among groups split by genotype (WT or Hu) and folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 24-52). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Kruskal-Wallis test for the following comparison: S, males vs. females of the same genotype and diet; F, comparisons of diets among the same sex and genotype.

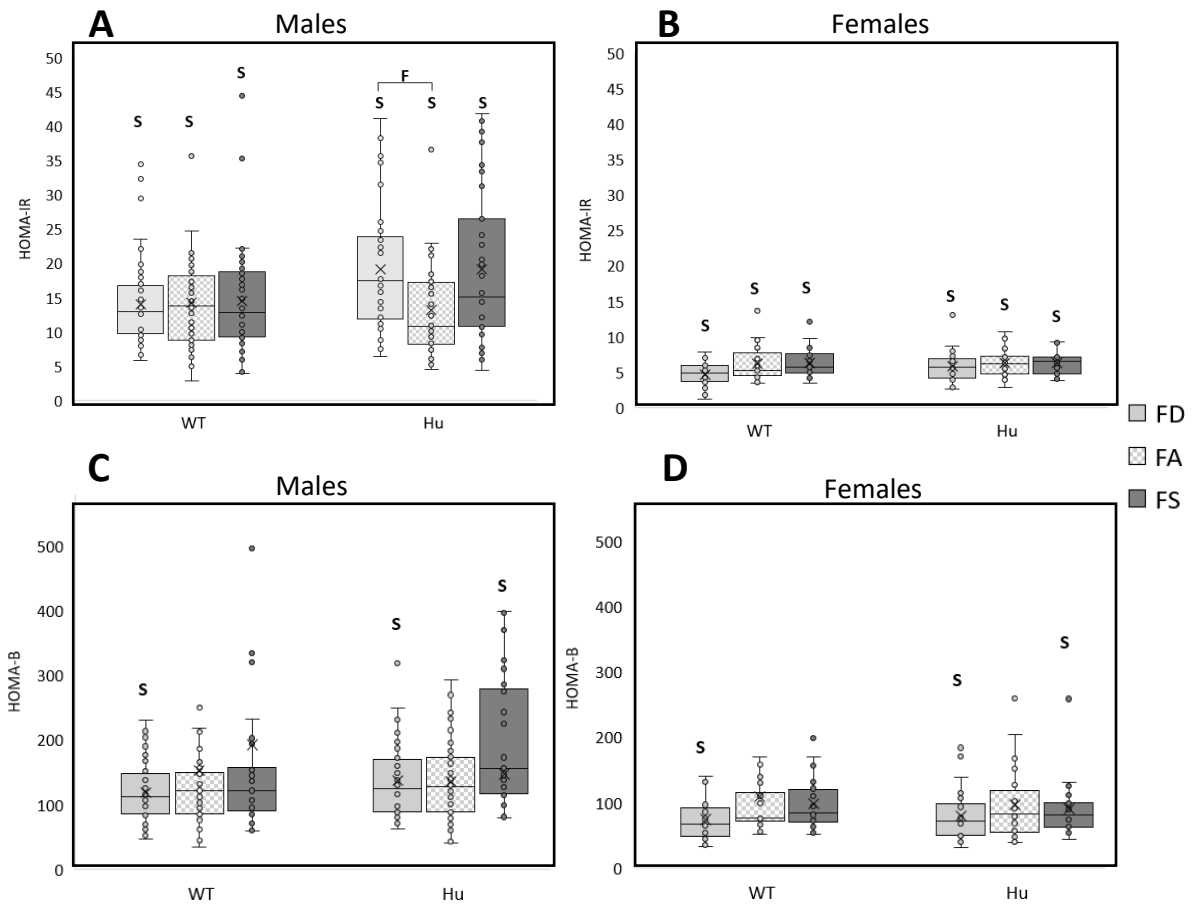

Figure 14: Pre iAs-exposure HOMA-IR and HOMA-B calculated from fasting blood glucose and plasma insulin collected after 6 weeks of consuming the modified folate diets. HOMA-IR in **(A)** males and **(B)** females and HOMA-B in **(C)** males and **(D)** females among groups split by genotype (WT or Hu) and folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 21-52). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparison: S, males vs. females of the same genotype and diet; F, comparisons of diets among the same sex and genotype.

Post iAs-exposure

Folate intake had no statistically significant effects on FBG regardless of sex, genotype, or the iAs exposure (**Figure 15 A, B**). As first seen at pre iAs-exposure, folate deficiency in Hu males resulted in higher FBG as compared to Hu males consuming the FA or FS diets, though differences were no longer statistically significant. WT and Hu males had higher post iAs-

exposure FPI than their female counterparts regardless of folate intake or iAs exposure ($p < 0.05$) (**Figure 15 C, D**). At post iAs-exposure, neither folate intake nor iAs exposure result in any significant differences in FPI regardless of sex or genotype.

Males had significantly higher HOMA-IR values than their female counterparts in control groups not exposed to iAs. Among those exposed to iAs, males tended to have higher HOMA-IR than females ($p < 0.05$), though differences not significant among WT mice on the FS diet or Hu mice on the FD diet (**Figure 16 A, B**). Males also tended to have higher HOMA-B values than females, though differences were not consistently significant across genotypes or iAs exposure levels (**Figure 16 C, D**). Folate intake, iAs exposure, and genotype did not result in significant differences in post iAs-exposure HOMA-IR or HOMA-B for males or females.

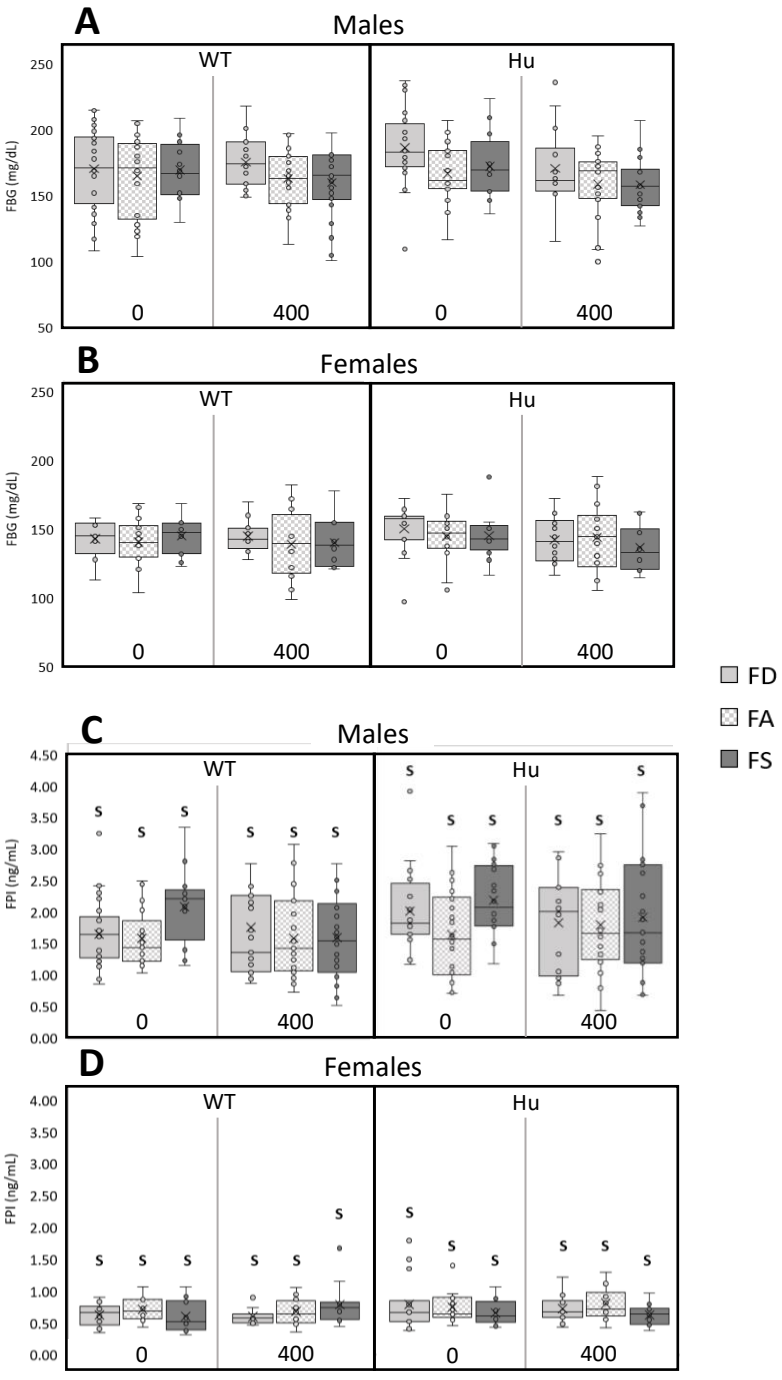

Figure 15: Post iAs-exposure fasting blood glucose and fasting plasma insulin after 11 weeks of consuming the modified folate diets with 5 weeks of exposure to 0 ppb or 400 ppb iAs. Fasting blood glucose in **(A)** males and **(B)** females and fasting plasma insulin in **(C)** males and **(D)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0ppb or400 ppb). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 12-28). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparisons: S, males vs. females of the same genotype and diet

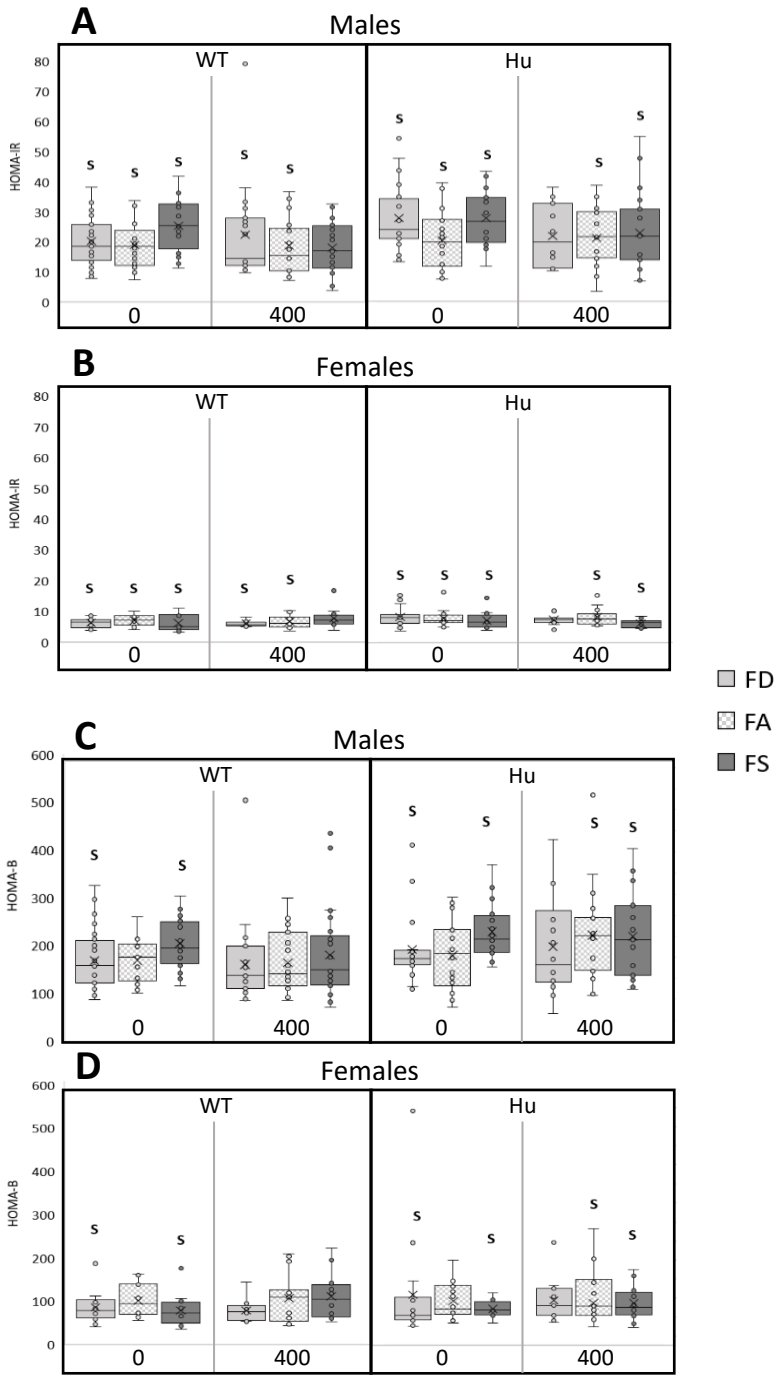

Figure 16: Post iAs-exposure HOMA-IR and HOMA-B calculated from fasting blood glucose and fasting plasma insulin collected after 11 weeks of consuming the modified folate diets with 5 weeks of exposure to 0 ppb or 400 ppb iAs. HOMA-IR in **(A)** males and **(B)** females and HOMA-B in **(C)** males and **(D)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0ppb or400 ppb). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 11-28). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Kruskal-Wallis test Welch unequal variance t-test for the following comparison: S, males vs. females of the same genotype and diet.

(Additional data on diabetes indicators, specifically on changes in FBG, FPI, HOMA-IR and HOMA-B from pre- to post iAs-exposure, are provided in the Appendix)

CHAPTER 4: DISCUSSION

I. Characterization of iAs Metabolism in Hu-C57BL/6N Mice

This study is the first to examine iAs metabolism in the recently established C57BL/6N mice carrying the human *AS3MT/BORCS7* locus. Hu males weighed more than WT males, a difference that was consistent through the entire study regardless of folate intake or iAs exposure. In our previous study using humanized 129S6 mice, the Hu males had lower bodyweights but higher percent fat mass than WT males (data unpublished – Miller 2021 Senior Honors Thesis). In this previous study WT-129S6 and Hu-129S6 mice were co-housed, therefore differences between the genotypes in food and water consumption could not be determined; additionally, mice that are co-housed share some of their gut microbiota, so differences in bodyweight can be interpreted without consideration of the potential role gut microflora can play. In the present study, mice of different genotypes (WT-C57BL/6N and Hu-C57BL/6N) were housed separately and water and food consumption per cage were measured regularly. We found that Hu males consumed more food and water per day than WT males which may explain the difference in bodyweight. However, as mice were not co-housed there could be metabolic effects from the gut microflora of the different genotypes that cannot be determined in the present study. The body composition of mice in the present study was not assessed, so differences in percent fat mass and percent lean mass could not be determined. The different genetic backgrounds of the mice used in these two studies (129S6 vs. C57BL/6N) may explain the different effect of humanizing *AS3MT* on the body weights of male mice. Previous studies have found that

C57BL/6 mice fed a low- or high-fat diet gain more weight and have lower fed metabolic rates than 129S6 mice (Almind & Kahn, 2004; Ussar et al., 2016). Therefore, humanizing the *AS3MT* gene in C57BL/6N mice may enhance the weight gain that is typically seen in the WT C57BL/6N mice while other mechanisms may be at play in 129S6 mice to increase fat mass while decreasing overall body weight. An additional consideration is that the Hu and WT (C57BL/6N) mice of the present study were bred in different facilities while the Hu and WT (129S6) mice in the Koller study were bred at the same facility which may also have effects on the gut microbiota in the mice (Franklin & Ericsson, 2017). Due to this difference in microbiota and the potential impacts on metabolism, we can make limited comparisons about the differences in bodyweight. Another consideration is that co-housed WT male mice (i.e., from Charles River) were notably more aggressive towards cage-mates compared to the Hu mice. The WT mice were from several litters that were weaned together, but the Hu mice within a cage were of the same litter. The aggression and associated stress among the WT males may have contributed to observed lower bodyweights compared to Hu male mice.

In the present study, the Hu mice excreted les tAs and exhibited higher %iAs and %MAs with lower %DMAs in urine compared to the WT mice. In WT mice fed the control (FA) diet, the urinary As profiles were characterized by greater than 88% DMAs, 0-2% MAs, and 0-11% iAs while in Hu mice the urinary tAs was represented by 17-50% DMAs, 10-31% MAs, and 31-55% iAs. The proportions of iAs and its methylated metabolites are similar to those seen in the previous study using Hu-129S6 mice (Koller et al., 2020). Koller and colleagues exposed WT-129S6 and Hu-129S6 mice to 400 ppb iAs for 4 weeks, after which the urinary arsenic profiles in WT mice were represented by greater than 97% DMAs with trace %MAs and %iAs while Hu mice had 48-55% DMAs with 5-11% MAs and 35-47% iAs (Koller et al., 2020). These urinary

As profiles indicate that Hu mice both on C57BL/6N and 129S6 backgrounds have a reduced capacity to methylate iAs.

The study by Koller and colleagues as well as previous unpublished research in our lab have found that the Hu-129S6 male mice excrete less total As in urine than WT-129S6 males when exposed to 400 ppb for 4 weeks (Koller et al., 2020) or 100 ppb iAs for 16 weeks (unpublished data – Miller 2021 Senior Honors Thesis). The same trends were observed in the present study at both the 0 ppb and 400 ppb iAs exposure groups fed the control (FA) diet.

Analysis of As species in livers of the Hu and WT mice performed in the present study also revealed that the Hu mice retained higher tAs in the liver compared to WT mice regardless of folate intake. Similar to what is seen in the urine, Hu mice had higher %iAs and lower %DMAs compared to WT mice. Among mice exposed to iAs, the livers of Hu mice fed the FA diet were characterized by 69-86% iAs, 12-25% MAs, and 78-93% DMAs while the livers of exposed WT mice were characterized by 4-12% iAs, 4-10% MAs, and 2-3% DMAs. The total concentration and relative proportions of arsenicals are similar to what was found by Koller and colleagues after exposing Hu-129S6 mice to 400 ppb iAs for 4 weeks. Previous studies reported that Hu-129S6 mice have more than 80% iAs and more than 15% MAs with only trace content of DMAs in the liver (Koller et al., 2020). Compared to Hu-129S6 mice, the Hu (C57BL/6N) mice in the present study appear to have increased methylation capacity for retained iAs due to the higher proportions of DMAs in the livers.

The lower tAs in urine and higher tAs in livers further reflects the reduced capacity to excrete iAs in Hu mice compared to WT mice, offering more evidence to the link between methylation as a key mechanism for detoxification of iAs.

II. Characterization of Diabetes Indicators in Hu-C57BL/6N Mice

The diabetic phenotypes assessed in this study included fasting blood glucose (FBG), fasting plasma insulin (FPI) as well as the metabolic indices HOMA-IR and HOMA-B. HOMA-IR was initially developed to determine insulin resistance in clinical settings for human subjects (Katsuki et al., 2001), though it is used here and as a proxy for insulin resistance in mice. At baseline (7-9 weeks of age), the Hu mice had higher FBG and lower FPI as compared to the WT mice, though this difference was only significant among males. The Hu females had lower HOMA-IR while both male and female Hu mice had lower HOMA-B than their WT counterparts. Together these suggest that differences exist between young Hu and WT mice in glucose metabolism and insulin sensitivity, but these differences are sex-dependent.

After 6 weeks of consuming the FA diet with an additional 5 weeks of 0 ppb or 400 ppb iAs exposure while on the same diet, there were no longer any genotype-related differences in FBG, FPI, or metabolic indices. After the first 6 weeks, the Hu males exhibited a larger increase in FPI with an increase in HOMA-B from baseline to pre iAs-exposure compared to a decreased HOMA-B seen in WT males. This indicates that the management of glucose metabolism required an increased secretion of insulin from pancreatic beta cells in aging Hu mice, while the opposite is true about the WT males. Five weeks of 400 ppb iAs exposure did not result in significant changes in any diabetic phenotypes among the Hu or WT males, nor were there sex-related differences in either genotype. In previously published studies using WT mice, prolonged exposure to iAs (8-20+) weeks or prenatal and *in utero* exposures were required for the development of diabetic phenotypes (Huang, Douillet, Dover, & Stýblo, 2018). The results of this study suggest that the 5 weeks of iAs exposure in adult mice may not be a long enough period of time for diabetic phenotypes to develop in either WT or Hu mice.

III. The Effect of Folate on iAs Metabolism and Diabetes Indicators

1. Modified folate intake alters folate status in mice

Male and female WT and Hu mice consumed an amino acid defined folate deficient (FD), folate adequate (FA), or folate supplemented (FS) diet for a total of 11 weeks. Plasma folate was measured at 6 and 11 weeks from the start of the study, i.e., before and after exposure to iAs. Standard rodent diets typically contain 2 mg folate/kg of diet as an "adequate" folate intake (Reeves, 1989; Reeves et al., 1993). The FD diet contained 0 mg folate/kg diet though mice were still able to absorb folate produced by their gut microbiota. We chose not to treat mice with an antibiotic to suppress bacterial folate production because adverse effects of the combined antibiotic and iAs treatment were observed in one of our previously published studies (Tsang et al., 2012). This approach explains why the mice consuming the FD diet had detectable folate levels in plasma. The differences in plasma folate due to folate intake were significant between all three diets for both WT and Hu males and females, with the single exception of WT females consuming FA vs FS diet where the difference did not reach significance. After 11 weeks of consuming the modified folate diets, there was a notable decrease in plasma folate among all groups regardless of iAs exposure. The differences in plasma folate due to folate intake were maintained from the pre iAs-exposure timepoint, though the differences in plasma folate between the FS and FA diets were generally more modest. The variation and absolute changes in plasma folate levels in mice consuming the FD diet were very small compared to mice consuming the FA or FS diets. Exposure to iAs typically resulted in a decrease in plasma folate level compared to non-exposed mice regardless of genotype or diet group, though these differences were often not statistically significant. Only among Hu males consuming the FA or FS diet and exposed to iAs was the decreased plasma folate statistically significant. Mice exposed to iAs while

consuming the FD or FA diets similarly tended to have lower plasma folate compared to non-exposed mice in another study (Huang, Douillet, Dover, Zhang, et al., 2018). Though the reductions in plasma folate due to iAs exposure were marginal both in the present study and in previous studies, it may indicate an increased demand for and consumption of folate for the purpose of SAM synthesis for iAs methylation. In the present study, it was observed that female mice were more resilient to change in plasma folate compared to males when consuming either the low or high folate diets. This has been observed in other studies, where folate deficiency and supplementation produce smaller changes in plasma folate compared to males (Huang, Douillet, Dover, Zhang, et al., 2018).

The reduction in plasma folate due to low folate intake (FD diet) is consistent with what has been observed in other studies (Speigelstein 2003, 2005). Erythrocyte and tissue folate can be better markers for folate availability in mammals while plasma folate will be more susceptible to modified folate intake (Kopp et al., 2017; Schmitz et al., 1994). For example, mice fed a reduced folate diet had a 6-fold decrease in plasma folate compared to controls, but only a 0.5-fold decrease in erythrocyte and liver folate in one of the previously published studies (Schmitz et al., 1994). Analysis of liver folate, which will be carried out in the near future is expected to provide additional insight to the actual folate availability in mice fed the modified folate diets.

To date, few studies have assessed any changes modified folate intake may have on the microbiome of mice. One study found that folate deficiency or supplementation did not result in alterations in the microflora of mice, though this was assessed in the context of colitis and colon cancer (MacFarlane et al., 2013). It would be beneficial to gauge if folate intake affects the growth of folate-producing bacteria to compensate for either deficient or excess folate availability. An investigation into the role of folate intake on changes in the gut microbiome may

be an interesting next step to understand the interaction between folate homeostasis and iAs metabolism.

2. Effects of folate intake on iAs metabolism

Folate is associated with once carbon metabolism and the synthesis of the universal methyl donor s-adenosylmethionine (SAM). SAM is essential for the metabolism of iAs by providing methyl groups for the subsequent methylation reactions catalyzed by AS3MT. Therefore, we expected to see effects of folate intake on iAs methylation and excretion in both WT and Hu mice. However, we found no statistically significant differences in tAs concentration or in the distribution of As species in spot urine from samples collected from the WT and Hu mice with different folate intakes. We only observed trends suggesting that increased folate intake with iAs exposure may facilitate excretion of tAs in urine of the Hu mice. This effect may become statistically significant after the remaining urine samples are analyzed. The trends in the proportions of arsenicals in urine suggest that reduced folate intake may decrease %MAs in a dose response manner among control, but not iAs-exposed Hu females.

Few laboratory studies have been conducted to date that examine specifically the effects of folate on iAs metabolism. Differences in designs and outcomes of these studies makes it difficult to conclusively characterize the role of folate in iAs methylation in laboratory animal models. In a study by Huang et al., WT mice exposed to iAs and fed a low fat diet with low folate content for 6 weeks tended to have lower tAs in urine compared to supplemented with folate, though differences were not statistically significant (Huang, Douillet, Dover, Zhang, et al., 2018). In contrast, studies in which a single oral dose of iAs was administered after feeding a low-folate diet tended to see significant decreases in tAs levels in urine compared to when the mice were fed an adequate folate intake (Spiegelstein et al., 2003, 2005). The exposure of mice

to a subchronic dose of iAs in the present study are consistent with the findings of Huang and colleagues, where tAs in urine was not significantly altered by folate intake.

We also expected to see effects of folate intake on the retention of iAs and on the methylation of retained iAs both WT and Hu mice. However, similar to the urine As profiles, we found no statistically significant differences tAs or the distribution of As species in livers of WT and Hu mice with different folate intakes. Studies conducted in rats have found that folate deficiency leads to upregulation in intestinal folate uptake mainly through the increased expression of folate transport proteins (Said et al., 2000; Wani et al., 2012). These adaptations to reduced folate intake may be sufficient to maintain normal body functions in rats and mice which could explain the lack of difference in iAs methylation capacity between FD and FA/FS diet groups in the present study. Though folate availability is reduced, it may be sufficient for the synthesis of SAM to support methylation of iAs at the given exposure levels. Analysis of As species and folate in the liver (to be performed in the near future) will help us answer these questions. For the present study, it would also be beneficial to measure SAM and SAH levels in plasma and liver to better assess the effects of folate intake on SAM availability for iAs metabolism.

3. Effects of folate intake on body weight and diabetic phenotypes

Folate is known to play a role in growth. Published data suggest that mice beginning a reduced-folate diet at 4 weeks of age and continuing to consume the diet for 12 weeks began to have lower bodyweights than mice fed a folate supplemented diet (Kopp et al., 2017). Therefore, to avoid potential negative outcomes of reduced folate on mouse growth and development in this study, we chose to begin the modified folate diets in adult mice. The only significant effect of modified folate intake was seen in Hu males, where folate supplementation resulted in

consistently higher body weights through the 11-week feeding period. Folate deficiency did not result in reduced bodyweights for mice in any dietary group.

Folate supplementation has commonly been associated with positive health outcomes such as reduction in neural tube defects. However, an over-supplementation of folate has been found to promote negative health outcomes such as metabolic dysregulation and T2D. Specifically, in pregnant mice, folic acid supplementation resulted in lower insulin synthesis, impaired insulin secretion, and impaired fat metabolism in offspring (Kintaka et al., 2020). However, folate deficiency has also been associated with T2D. Patients with T2D have lower folate and higher homocysteine in serum than healthy individuals (Al-Maskari et al., 2012; Wang et al., 2017). In the present study, we found that folate deficiency in Hu males was associated with higher FBG and HOMA-IR compared to Hu males consuming the FA and FS diets; folate deficiency also resulted in increased FPI, though the differences between mice fed the FD, FA, or FS diet were not statistically significant. Combined, these findings may indicate that folate deficiency exacerbates insulin resistance in Hu males. However, there were no indications of the development of diabetic phenotypes due to folate supplementation among males or females in this study.

IV. Folate Metabolism in Mice Compared to Humans: Implications and Limitations

Mouse models are commonly used to study metabolic diseases as well as the effects of altered nutrient intake on health outcomes. Though the role of folate intake on As metabolism is often studied in mice, it is important to consider species differences in folate metabolism. One major difference between mouse and human folate metabolism is in *FPGS*, the gene encoding folylpolyglutamate synthetase (FPGS). FPGS catalyzes the reaction that adds glutamate residues to folates when they enter a cell and is therefore critical for the retention of folates in cells.

Mouse *Fpgs* has two promoters, P1 which is used extensively in the liver and kidney, and P2 which is similar to the human promoter and is expressed at low levels in most tissues (Turner et al., 1999). Only when P1 is deleted do mice express *Fpgs* in similar patterns as humans (Yang et al., 2014). Both the Hu and WT mice in the present study have unaltered mouse *Fpgs* which may explain why folate deficiency did not result in reduced capacity to methylate (i.e., in changes in proportions of urinary As species) that is commonly found in human studies.

V. Conclusions

The results of this study indicate that Hu-C57BL/6N background are good models for human iAs methylation capacity illustrated by the decreased proportion of fully methylated iAs metabolites both retained in the livers and excreted in the urine. While the Hu mice tended to excrete less tAs and retain more tAs than the WT mice, more work will need to be completed to quantify the retention of iAs species in various tissues of Hu mice to further assess if this model is suitable to estimate human iAs metabolism across other tissues.

The Hu mice did not exhibit an increased susceptibility to diabetic phenotypes than the WT mice with 5 weeks of iAs exposure. More work should be done with longer exposure windows to further investigate the suitability of Hu mice to model humans in the development of T2D due to chronic iAs exposure.

While modified folate intake with iAs exposure resulted in trends in tAs in urine of the Hu mice indicating folate supplementation increases tAs, there was no evidence in the urinary As profiles that folate intake altered the iAs methylation capacity either the Hu or WT mice. The Hu mice do not appear to be the best model for humans to reflect the effects of folate in iAs metabolism. Improvement of this model could involve humanizing the *FPGS* gene in mice in

combination with the Hu *AS3MT/BORCS7* locus, though further research would need to confirm this hypothesis.

The work associated with this study continues and will include quantification of hepatic folate, SAM, and SAH to better determine the methyl donor status in control and iAs-exposed mice consuming diets with modified folate content. This work will also focus on analysis of tAs content and proportions of iAs and its methylated metabolites in various tissues such as kidney, pancreas, and spleen among others. Results of this work will provide additional information about the differences in iAs metabolism between the Hu and WT mice and the interaction between folate intake and iAs metabolism.

APPENDIX: CHANGES IN DIABETES PHENOTYPES AND METABOLIC INDICES

Change from Baseline to Pre iAs-Exposure

Overall, males tended to have a greater increase in FBG and FPI from baseline to pre iAs-exposure compared to females, though differences were not always significant (**Figure 17**). Among WT mice, males had a greater increase in FBG from baseline to pre iAs-exposure than their female counterparts for all diet groups ($p < 0.05$). In contrast, only Hu males consuming the FD diet had a greater increase in FBG than females ($P < 0.05$). Among males, Hu mice consuming the FA and FS diets had a smaller increase in FBG from baseline to pre iAs-exposure compared to their WT counterparts ($p < 0.05$), though this was not observed in mice consuming the FD diet. Folate intake significantly affected the change in FBG among Hu males only, where mice consuming the FD diet had a larger increase in FBG than those consuming the FA diet ($p < 0.05$). There were no significant differences in the change in FBG among female mice regardless of genotype or folate intake.

WT males consuming the FD diet had a greater increase in FPI compared to females ($p < 0.05$); these sex-related differences were also seen in WT mice consuming the FA and FS diet groups, though differences were not statistically significant. In contrast, Hu males had significantly greater increases in FPI compared to Hu females for all diet groups ($p < 0.05$). In both males and females, folate deficiency resulted in a greater change in FPI in Hu mice compared to their WT counterparts ($p < 0.05$).

WT and Hu males had a larger increase in HOMA-IR compared to their female counterparts for all diet groups ($p < 0.05$) (**Figure 18 A, B**). Hu mice consuming the FD diet had a larger increase in HOMA-IR compared to WT counterparts ($p < 0.05$). Among males, Hu mice

exhibited a significantly larger increase in HOMA-B in all diet groups compared to their WT counterparts ($p < 0.05$) (**Figure 18 C, D**). There were no differences in the change in HOMA-B among female mice due to genotype or folate intake. Only among Hu mice consuming the FD diet, males had a significantly greater increase in HOMA-B compared to Hu females ($p < 0.05$).

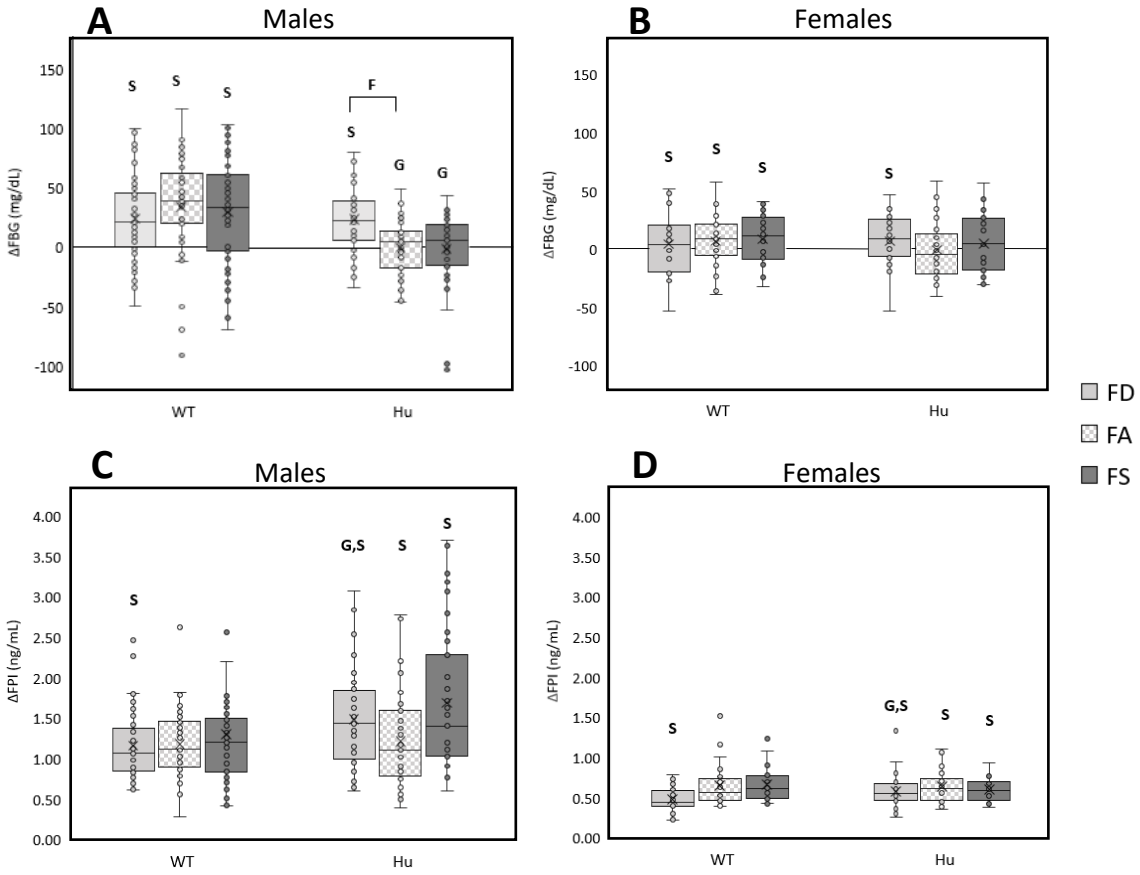

Figure 17: Change in fasting blood glucose and fasting plasma from baseline to pre iAs-exposure. Changes in fasting blood glucose in **(A)** males and **(B)** females and changes in fasting plasma insulin in **(C)** males and **(D)** females among groups split by genotype (WT or Hu) and folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 24-52). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparisons: G, WT vs. Hu of the same sex and diet; S, males vs. females of the same genotype and diet; F, comparisons of diets among the same sex and genotype.

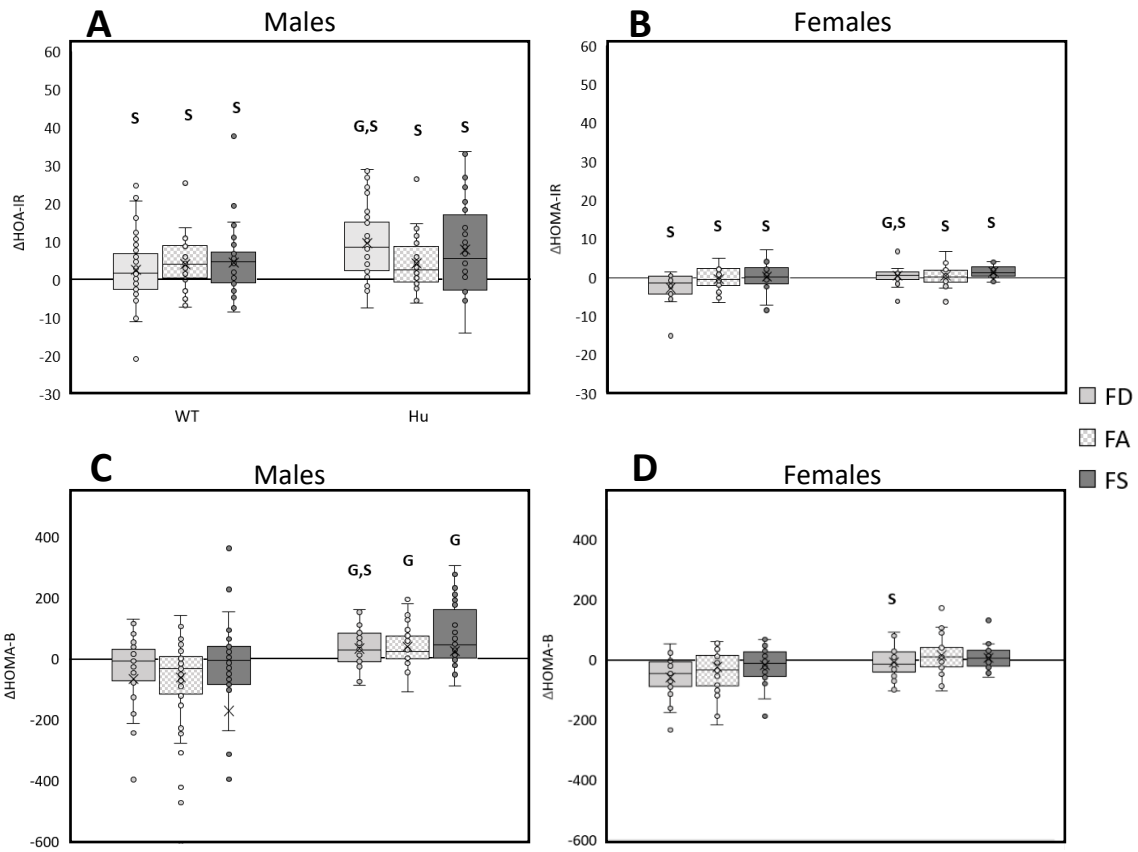

Figure 18: Change in HOMA-IR and HOMA-B from baseline to pre iAs-exposure calculated from fasting blood glucose and fasting plasma insulin at each timepoint. Change in HOMA-IR in **(A)** males and **(B)** females and HOMA-B in **(C)** males and **(D)** females among groups split by genotype (WT or Hu) and folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 24-52). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles; HOMA-IR values greater than 500 and less than -500 are not shown. $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparisons: G, WT vs. Hu of the same sex and diet; S, males vs. females of the same genotype and diet.

Change from Pre iAs-Exposure to Post iAs-Exposure

There were no clear trends in the change in FBG from pre iAs-exposure to post iAs-exposure among WT or Hu mice due to folate intake or iAs exposure, regardless of sex (**Figure 19 A, B**). Among non-exposed mice consuming the FS diet, females tended to have a greater increase in FBG compared to males, though differences were only significant among WT mice ($p < 0.05$). In contrast, among mice exposed to iAs and consuming the FS diet, males tended to

have a greater increase in FBG among males compared to females, though differences were only significant among Hu mice ($p < 0.05$).

Males tended to have a greater increase in FPI from pre iAs-exposure to post iAs-exposure compared to females, though differences were not always statistically significant (**Figure 19 C, D**). In general, males had a greater change in HOMA-IR from pre iAs-exposure to post iAs-exposure than their female counterparts, though statistically significant differences were only observed among mice exposed to iAs ($p < 0.05$) (**Figure 20 A, B**). Exposure to iAs had inconsistent effects on the change in HOMA-IR among male mice. iAs exposure resulted in a significantly smaller change in HOMA-IR when combined with the FD diet in Hu males and with consumption of the FA and FS diet in WT males ($p < 0.05$). Exposure to iAs did not result in any significant differences in the change in HOMA-B in mice regardless of sex, genotype, or diet (**Figure 20 C, D**). Males tended to have a greater increase in HOMA-B than their female counterparts, though differences were not consistently significant.

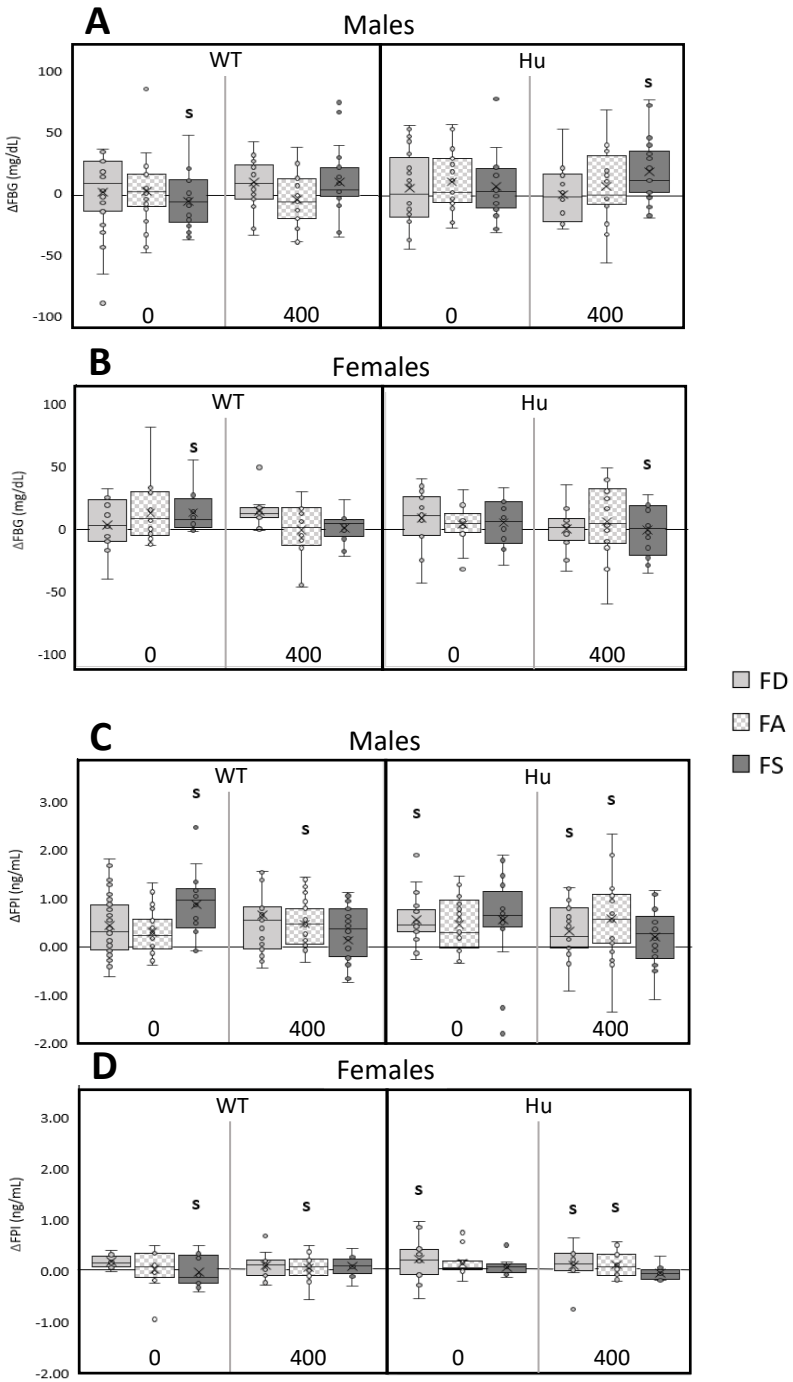

Figure 19: Change in fasting blood glucose and fasting plasma insulin from pre iAs-exposure to post iAs-exposure. Change in fasting blood glucose in **(A)** males and **(B)** females and change in fasting plasma insulin in **(C)** males and **(D)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0ppb or 400 ppb). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 9-28). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Welch unequal variance t-test for the following comparison: S, males vs. females of the same genotype, diet, and iAs exposure.

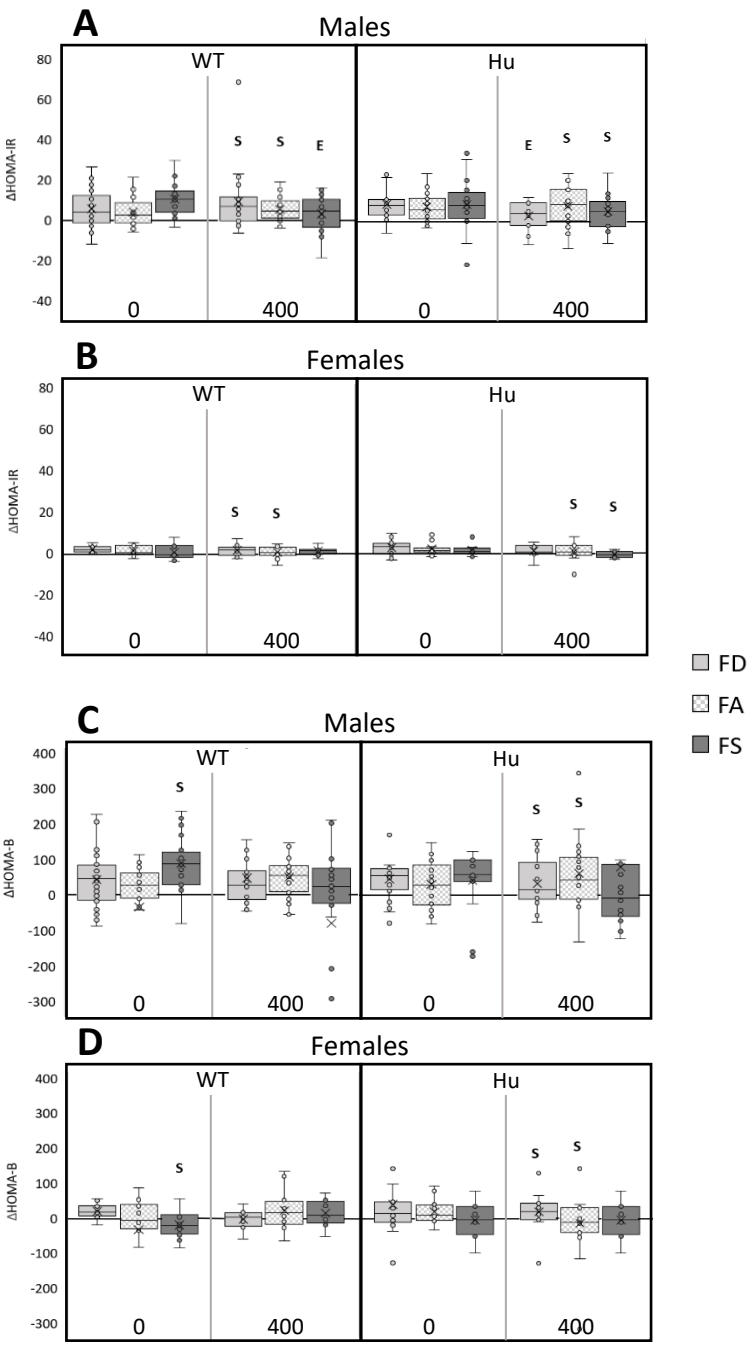

Figure 20: Change in HOMA-IR and HOMA-B from pre iAs-exposure to post iAs-exposure calculated from fasting blood glucose and fasting plasma insulin collected at each timepoint. HOMA-IR in **(A)** males and **(B)** females and HOMA-B in **(C)** males and **(D)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0ppb or 400 ppb). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 8-28). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles; values greater than 300 and less than -300 are excluded from the graphs. $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparisons: S, males vs. females of the same genotype, diet, and iAs exposure; E, 0 ppb vs. 400 ppb of the same sex, genotype, and diet.

Change from Baseline to Post iAs-Exposure

Males tended to have a greater increase in FBG from baseline to post iAs-exposure in all diet groups compared to their female counterparts, regardless of genotype or iAs exposure, though differences were typically not statistically significant (**Figure 21 A, B**). There were no significant differences in the change in FBG due to genotype.

WT and Hu males tended to have a greater increase in FPI from baseline to post iAs-exposure compared to females in all diet groups. These differences were significant among Hu mice regardless of diet or iAs-exposure and among WT males not exposed to iAs ($p < 0.05$) (**Figure 21 C, D**).

WT and Hu males had a larger increase in HOMA-IR from baseline to post iAs-exposure than their female counterparts for all diet groups, regardless of iAs exposure ($p < 0.05$) (**Figure 22 A, B**). There were no significant differences in the change in HOMA-IR among male or female mice due to genotype or folate intake. Among males, exposure to iAs tended to result in a smaller increase in HOMA-IR compared to non-exposed counterparts, though differences were only significant among WT mice when consuming the FS diet ($p < 0.05$).

WT and Hu males tended to have an increase in HOMA-B from baseline to post iAs-exposure while WT and Hu females tended to have a decrease in HOMA-B in all diet groups (**Figure 22 C, D**). Differences in the change in HOMA-B between males and females was only significant among Hu mice ($p<0.05$). iAs exposure resulted in significantly greater increases in HOMA-B in Hu males compared to Hu females in all diet groups ($p < 0.05$). Hu males consuming the FS diet had a significantly greater increase in HOMA-B compared to Hu females, regardless of iAs exposure ($p < 0.05$). Hu male and female mice tended to have a greater increase

in HOMA-B from baseline to post iAs-exposure compared to their WT counterparts for all diet groups, though differences were only significant in iAs-exposed males consuming the FA diet and in non-exposed females consuming the FA diet ($p < 0.05$).

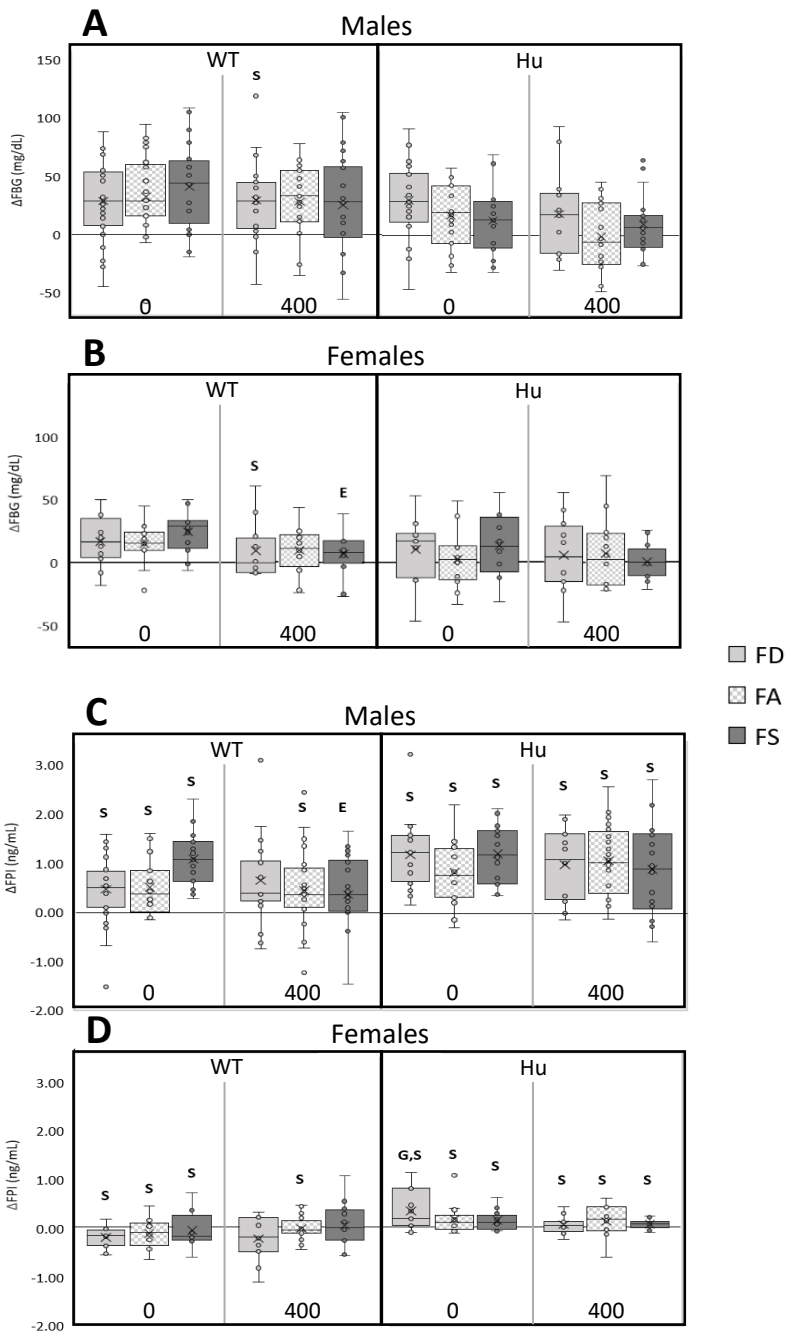

Figure 21: Change in fasting blood glucose and fasting plasma insulin from baseline to post iAs-exposure. Change in fasting blood glucose in **(A)** males and **(B)** females and change in fasting plasma insulin in **(C)** males and **(D)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0ppb or 400 ppb). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 12-27). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles. $p < 0.05$ using Kruskal-Wallis test for the following comparisons: G, WT vs. Hu of the same sex, diet, and iAs exposure. $p < 0.05$ using Welch unequal variance t-test or Mann-Whitney U-test (for non-normal distributions) for the following comparison: S, males vs. females of the same genotype, diet, and iAs exposure.

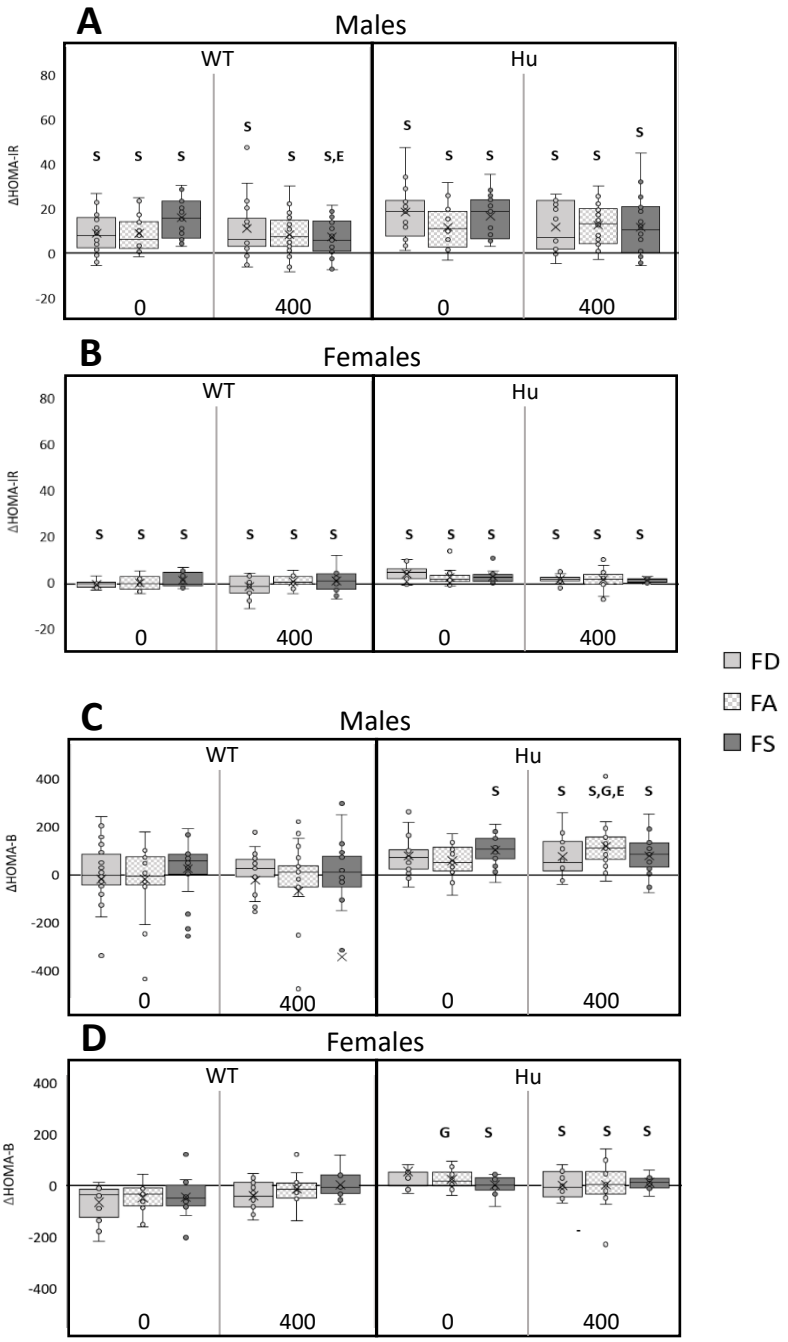

Figure 22: Change in HOMA-IR and HOMA-B from baseline to post iAs-exposure calculated from fasting blood glucose and fasting plasma insulin collected at each timepoint. HOMA-IR in **(A)** males and **(B)** females and HOMA-B in **(C)** males and **(D)** females among groups split by genotype (WT or Hu), folate intake (folate deficient, FD; folate adequate, FA; or folate supplemented, FS), and iAs exposure (0ppb or 400 ppb). Means are denoted by × and medians are denoted by the middle horizontal bar for each group (n = 11-28). First and third quartiles are represented by the box boundaries, and minimum and maximum values represented by the capped lines (whiskers) extending from the box. Individual data points are represented by circles; values greater than 300 and less than -300 are excluded from the graphs. $p < 0.05$ using Kruskal-Wallis test or Welch unequal variance t-test for the following comparisons: G, WT vs. Hu of the same sex, diet, and iAs exposure; S, males vs. females of the same genotype, diet, and iAs exposure; E, 0 ppb vs. 400 ppb of the same sex, genotype, and diet.

REFERENCES

129S6. (n.d.). Taconic Biosciences, Inc. Retrieved February 3, 2022, from https://www.taconic.com/mouse-model/129s6

000664—B6 Strain Details. (n.d.). Retrieved February 3, 2022, from https://www.jax.org/strain/000664

Acharyya, N., Deb, B., Chattopadhyay, S., & Maiti, S. (2015). Arsenic-Induced Antioxidant Depletion, Oxidative DNA Breakage, and Tissue Damages are Prevented by the Combined Action of Folate and Vitamin B12. *Biological Trace Element Research*, *168*(1), 122–132. https://doi.org/10.1007/s12011-015-0324-5

Al-Maskari, M. Y., Waly, M. I., Ali, A., Al-Shuaibi, Y. S., & Ouhtit, A. (2012). Folate and vitamin B12 deficiency and hyperhomocysteinemia promote oxidative stress in adult type 2 diabetes. *Nutrition*, *28*(7), e23–e26. https://doi.org/10.1016/j.nut.2012.01.005

Almind, K., & Kahn, C. R. (2004). Genetic determinants of energy expenditure and insulin resistance in diet-induced obesity in mice. *Diabetes*, *53*(12), 3274–3285. https://doi.org/10.2337/diabetes.53.12.3274

Bailey, L. B., & Caudill, M. A. (2012). Folate. In *Present Knowledge in Nutrition* (pp. 321–342). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119946045.ch21

Basu, A., Mitra, S., Chung, J., Guha Mazumder, D. N., Ghosh, N., Kalman, D., von Ehrenstein, O. S., Steinmaus, C., Liaw, J., & Smith, A. H. (2011). Creatinine, Diet, Micronutrients, and Arsenic Methylation in West Bengal, India. *Environmental Health Perspectives*, *119*(9), 1308–1313. https://doi.org/10.1289/ehp.1003393

Bhattacharjee, S., Sarkar, C., & Pal, S. (2013). Additive beneficial effect of folic acid and vitamin B12 co-administration on arsenic-induced oxidative damage in cardiac tissue In Vivo. *Asian Journal of Pharmaceutical and Clinical Research*, *6*.

Birling, M.-C., Yoshiki, A., Adams, D. J., Ayabe, S., Beaudet, A. L., Bottomley, J., Bradley, A., Brown, S. D. M., Bürger, A., Bushell, W., Chiani, F., Chin, H.-J. G., Christou, S., Codner, G. F., DeMayo, F. J., Dickinson, M. E., Doe, B., Donahue, L. R., Fray, M. D., … Murray, S. A. (2021). A resource of targeted mutant mouse lines for 5,061 genes. *Nature Genetics*, *53*(4), 416–419. https://doi.org/10.1038/s41588-021-00825-y

Bozack, A. K., Hall, M. N., Liu, X., Ilievski, V., Lomax-Luu, A. M., Parvez, F., Siddique, A. B., Shahriar, H., Uddin, M. N., Islam, T., Graziano, J. H., & Gamble, M. V. (2019). Folic acid supplementation enhances arsenic methylation: Results from a folic acid and creatine supplementation randomized controlled trial in Bangladesh. *The American Journal of Clinical Nutrition*, *109*(2), 380–391. https://doi.org/10.1093/ajcn/nqy148

Bozack, A. K., Howe, C. G., Hall, M. N., Liu, X., Slavkovich, V., Ilievski, V., Lomax-Luu, A. M., Parvez, F., Siddique, A. B., Shahriar, H., Uddin, M. N., Islam, T., Graziano, J. H., & Gamble, M. V. (2021). Betaine and choline status modify the effects of folic acid and creatine

supplementation on arsenic methylation in a randomized controlled trial of Bangladeshi adults. *European Journal of Nutrition*, *60*(4), 1921–1934. https://doi.org/10.1007/s00394-020-02377-z

Challenger, F. (1947). Biological methylation. *Science Progress*, *35*(139), 396–416.

Cichowicz, D. J., & Shane, B. (1987). Mammalian folylpoly-.gamma.-glutamate synthetase. 2. Substrate specificity and kinetic properties. *Biochemistry*, *26*(2), 513–521. https://doi.org/10.1021/bi00376a025

Cook, J. D., Cichowicz, D. J., George, S., Lawler, A., & Shane, B. (1987). Mammalian folylpoly-.gamma.-glutamate synthetase. 4. In vitro and in vivo metabolism of folates and analogues and regulation of folate homeostasis. *Biochemistry*, *26*(2), 530–539. https://doi.org/10.1021/bi00376a027

Douillet, C., Huang, M. C., Saunders, R. J., Dover, E. N., Zhang, C., & Stýblo, M. (2017). Knockout of arsenic (+3 oxidation state) methyltransferase is associated with adverse metabolic phenotype in mice: The role of sex and arsenic exposure. *Archives of Toxicology*, *91*(7), 2617–2627. https://doi.org/10.1007/s00204-016-1890-9

Drobna, Z., Narenmandura, H., Kubachka, K. M., Edwards, B. C., Herbin-Davis, K., Styblo, M., Le, X. C., Creed, J. T., Maeda, N., Hughes, M. F., & Thomas, D. J. (2009). Disruption of the arsenic (+3 oxidation state) methyltransferase gene in the mouse alters the phenotype for methylation of arsenic and affects distribution and retention of orally administered arsenate. *Chemical Research in Toxicology*, *22*(10), 1713–1720. https://doi.org/10.1021/tx900179r

El-Masri, H. A., & Kenyon, E. M. (2008). Development of a human physiologically based pharmacokinetic (PBPK) model for inorganic arsenic and its mono- and di-methylated metabolites. *Journal of Pharmacokinetics and Pharmacodynamics*, *35*(1), 31–68. https://doi.org/10.1007/s10928-007-9075-z

Fontaine, D. A., & Davis, D. B. (2016). Attention to Background Strain Is Essential for Metabolic Research: C57BL/6 and the International Knockout Mouse Consortium. *Diabetes*, *65*(1), 25–33. https://doi.org/10.2337/db15-0982

Franklin, C. L., & Ericsson, A. C. (2017). Microbiota and reproducibility of rodent models. *Lab Animal*, *46*(4), 114–122. https://doi.org/10.1038/laban.1222

Freeman, H. C., Hugill, A., Dear, N. T., Ashcroft, F. M., & Cox, R. D. (2006). Deletion of Nicotinamide Nucleotide Transhydrogenase: A New Quantitive Trait Locus Accounting for Glucose Intolerance in C57BL/6J Mice. *Diabetes*, *55*(7), 2153–2156. https://doi.org/10.2337/db06-0358

Gamble, M. V., Liu, X., Ahsan, H., Pilsner, J. R., Ilievski, V., Slavkovich, V., Parvez, F., Chen, Y., Levy, D., Factor-Litvak, P., & Graziano, J. H. (2006). Folate and arsenic metabolism: A double-blind, placebo-controlled folic acid–supplementation trial in Bangladesh. *American Journal of Clinical Nutrition*, *84*(5), 1093–1101. https://doi.org/10.1093/ajcn/84.5.1093

Gamble, M. V., Liu, X., Ahsan, H., Pilsner, J. R., Ilievski, V., Slavkovich, V., Parvez, F., Levy, D., Factor-Litvak, P., & Graziano, J. H. (2005). Folate, Homocysteine, and Arsenic Metabolism in Arsenic-Exposed Individuals in Bangladesh. *Environmental Health Perspectives*, *113*(12), 1683–1688. https://doi.org/10.1289/ehp.8084

Gamble, M. V., Liu, X., Slavkovich, V., Pilsner, J. R., Ilievski, V., Factor-Litvak, P., Levy, D., Alam, S., Islam, M., Parvez, F., Ahsan, H., & Graziano, J. H. (2007). Folic acid supplementation lowers blood arsenic. *The American Journal of Clinical Nutrition*, *86*(4), 1202–1209.

Howe, C. G., Niedzwiecki, M. M., Hall, M. N., Liu, X., Ilievski, V., Slavkovich, V., Alam, S., Siddique, A. B., Graziano, J. H., & Gamble, M. V. (2014). Folate and Cobalamin Modify Associations between S-adenosylmethionine and Methylated Arsenic Metabolites in Arsenic-Exposed Bangladeshi Adults123. *The Journal of Nutrition*, *144*(5), 690–697. https://doi.org/10.3945/jn.113.188789

Huang, M. C., Douillet, C., Dover, E. N., & Stýblo, M. (2018). Prenatal arsenic exposure and dietary folate and methylcobalamin supplementation alter the metabolic phenotype of C57BL/6J mice in a sex-specific manner. *Archives of Toxicology*, *92*(6), 1925–1937. https://doi.org/10.1007/s00204-018-2206-z

Huang, M. C., Douillet, C., Dover, E. N., Zhang, C., Beck, R., Tejan, -Sie Ahmad, Krupenko, S. A., & St, ýblo M. (2018). Metabolic Phenotype of Wild-Type and As3mt-Knockout C57BL/6J Mice Exposed to Inorganic Arsenic: The Role of Dietary Fat and Folate Intake. *Environmental Health Perspectives*, *126*(12), 127003. https://doi.org/10.1289/EHP3951

Huang, M. C., Douillet, C., Su, M., Zhou, K., Wu, T., Chen, W., Galanko, J. A., Drobná, Z., Saunders, R. J., Martin, E., Fry, R. C., Jia, W., & Stýblo, M. (2017). Metabolomic profiles of arsenic (+3 oxidation state) methyltransferase knockout mice: Effect of sex and arsenic exposure. *Archives of Toxicology*, *91*(1), 189–202. https://doi.org/10.1007/s00204-016-1676-0

Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline. (1998). *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK114310/

Katsuki, A., Sumida, Y., Gabazza, E. C., Murashima, S., Furuta, M., Araki-Sasaki, R., Hori, Y., Yano, Y., & Adachi, Y. (2001). Homeostasis Model Assessment Is a Reliable Indicator of Insulin Resistance During Follow-up of Patients With Type 2 Diabetes. *Diabetes Care*, *24*(2), 362–365. https://doi.org/10.2337/diacare.24.2.362

Kintaka, Y., Wada, N., Shioda, S., Nakamura, S., Yamazaki, Y., & Mochizuki, K. (2020). Excessive folic acid supplementation in pregnant mice impairs insulin secretion and induces the expression of genes associated with fatty liver in their offspring. *Heliyon*, *6*(4), e03597. https://doi.org/10.1016/j.heliyon.2020.e03597

Koller, B. H., Snouwaert, J. N., Douillet, C., Jania, L. A., El-Masri, H., Thomas, D. J., & Stýblo, M. (2020). Arsenic Metabolism in Mice Carrying a BORCS7/AS3MT Locus Humanized by Syntenic Replacement. *Environmental Health Perspectives*, *128*(8). https://doi.org/10.1289/EHP6943

Kopp, M., Morisset, R., & Rychlik, M. (2017). Characterization and Interrelations of One-Carbon Metabolites in Tissues, Erythrocytes, and Plasma in Mice with Dietary Induced Folate Deficiency. *Nutrients*, *9*(5), E462. https://doi.org/10.3390/nu9050462

Kurzius-Spencer, M., da Silva, V., Thomson, C. A., Hartz, V., Hsu, C.-H., Burgess, J. L., O'Rourke, M. K., & Harris, R. B. (2017). Nutrients in one-carbon metabolism and urinary arsenic methylation in the National Health and Nutrition Examination Survey (NHANES) 2003–2004. *Science of The Total Environment*, *607–608*, 381–390. https://doi.org/10.1016/j.scitotenv.2017.07.019

Lin, S., Shi, Q., Nix, F. B., Styblo, M., Beck, M. A., Herbin-Davis, K. M., Hall, L. L., Simeonsson, J. B., & Thomas, D. J. (2002). A novel S-adenosyl-L-methionine:arsenic(III) methyltransferase from rat liver cytosol. *The Journal of Biological Chemistry*, *277*(13), 10795–10803. https://doi.org/10.1074/jbc.M110246200

López-Carrillo, L., Gamboa-Loira, B., Becerra, W., Hernández-Alcaraz, C., Hernández-Ramírez, R. U., Gandolfi, A. J., Franco-Marina, F., & Cebrián, M. E. (2016). Dietary micronutrient intake and its relationship with arsenic metabolism in Mexican women. *Environmental Research*, *151*, 445–450. https://doi.org/10.1016/j.envres.2016.08.015

MacFarlane, A. J., Behan, N. A., Matias, F. M. G., Green, J., Caldwell, D., & Brooks, S. P. J. (2013). Dietary folate does not significantly affect the intestinal microbiome, inflammation or tumorigenesis in azoxymethane–dextran sodium sulphate-treated mice. *British Journal of Nutrition*, *109*(4), 630–638. https://doi.org/10.1017/S0007114512001857

McDorman, E. W., Collins, B. W., & Allen, J. W. (2002). Dietary folate deficiency enhances induction of micronuclei by arsenic in mice. *Environmental and Molecular Mutagenesis*, *40*(1), 71–77. https://doi.org/10.1002/em.10085

Mekada, K., Abe, K., Murakami, A., Nakamura, S., Nakata, H., Moriwaki, K., Obata, Y., & Yoshiki, A. (2009). Genetic differences among C57BL/6 substrains. *Experimental Animals*, *58*(2), 141–149. https://doi.org/10.1538/expanim.58.141

Niedzwiecki, M. M., Hall, M. N., Liu, X., Slavkovich, V., Ilievski, V., Levy, D., Alam, S., Siddique, A. B., Parvez, F., Graziano, J. H., & Gamble, M. V. (2014). Interaction of plasma glutathione redox and folate deficiency on arsenic methylation capacity in Bangladeshi adults. *Free Radical Biology & Medicine*, *0*, 67–74. https://doi.org/10.1016/j.freeradbiomed.2014.03.042

Peters, B. A., Hall, M. N., Liu, X., Parvez, F., Sanchez, T. R., van Geen, A., Mey, J. L., Siddique, A. B., Shahriar, H., Uddin, M. N., Islam, T., Balac, O., Ilievski, V., Factor-Litvak, P., Graziano, J. H., & Gamble, M. V. (2015). Folic Acid and Creatine as Therapeutic Approaches to

Lower Blood Arsenic: A Randomized Controlled Trial. *Environmental Health Perspectives*, *123*(12), 1294–1301. https://doi.org/10.1289/ehp.1409396

Pilsner, J. R., Liu, X., Ahsan, H., Ilievski, V., Slavkovich, V., Levy, D., Factor-Litvak, P., Graziano, J. H., & Gamble, M. V. (2009). Folate Deficiency, Hyperhomocysteinemia, Low Urinary Creatinine, and Hypomethylation of Leukocyte DNA Are Risk Factors for Arsenic-Induced Skin Lesions. *Environmental Health Perspectives*, *117*(2), 254–260. https://doi.org/10.1289/ehp.11872

Reeves, P. G. (1989). AIN-76 diet: Should we change the formulation? *The Journal of Nutrition*, *119*(8), 1081–1082. https://doi.org/10.1093/jn/119.8.1081

Reeves, P. G., Nielsen, F. H., & Fahey, G. C. (1993). AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *The Journal of Nutrition*, *123*(11), 1939–1951. https://doi.org/10.1093/jn/123.11.1939

Said, H. M., Chatterjee, N., Haq, R. U., Subramanian, V. S., Ortiz, A., Matherly, L. H., Sirotnak, F. M., Halsted, C., & Rubin, S. A. (2000). Adaptive regulation of intestinal folate uptake: Effect of dietary folate deficiency. *American Journal of Physiology. Cell Physiology*, *279*(6), C1889-1895. https://doi.org/10.1152/ajpcell.2000.279.6.C1889

Schmitz, J. C., Grindey, G. B., Schultz, R. M., & Priest, D. G. (1994). Impact of dietary folic acid on reduced folates in mouse plasma and tissues. Relationship to dideazatetrahydrofolate sensitivity. *Biochemical Pharmacology*, *48*(2), 319–325. https://doi.org/10.1016/0006-2952(94)90103-1

Sijko, M., & Kozłowska, L. (2021). Influence of Dietary Compounds on Arsenic Metabolism and Toxicity. Part II—Human Studies. *Toxics*, *9*(10), 259. https://doi.org/10.3390/toxics9100259

Spiegelstein, O., Lu, X., Le, X. C., Troen, A., Selhub, J., Melnyk, S., James, S. J., & Finnell, R. H. (2003). Effects of dietary folate intake and folate binding protein-1 (Folbp1) on urinary speciation of sodium arsenate in mice. *Toxicology Letters*, *145*(2), 167–174. https://doi.org/10.1016/S0378-4274(03)00307-2

Spiegelstein, O., Lu, X., Le, X. C., Troen, A., Selhub, J., Melnyk, S., James, S. J., & Finnell, R. H. (2005). Effects of dietary folate intake and folate binding protein-2 (Folbp2) on urinary speciation of sodium arsenate in mice. *Environmental Toxicology and Pharmacology*, *19*(1), 1–7. https://doi.org/10.1016/j.etap.2004.01.007

Spratlen, M. J., Gamble, M. V., Grau-Perez, M., Kuo, C.-C., Best, L. G., Yracheta, J., Francesconi, K., Goessler, W., Mossavar-Rahmani, Y., Hall, M., Umans, J. G., Fretts, A., & Navas-Acien, A. (2017). Arsenic metabolism and one-carbon metabolism at low-moderate arsenic exposure: Evidence from the Strong Heart Study. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *105*, 387–397. https://doi.org/10.1016/j.fct.2017.05.004

Stevens, C. E., & Hume, I. D. (1998). Contributions of Microbes in Vertebrate Gastrointestinal Tract to Production and Conservation of Nutrients. *Physiological Reviews*, *78*(2), 393–427. https://doi.org/10.1152/physrev.1998.78.2.393

Stýblo, M., Douillet, C., Bangma, J., Eaves, L. A., de Villena, F. P.-M., & Fry, R. (2019). Differential metabolism of inorganic arsenic in mice from genetically diverse Collaborative Cross strains. *Archives of Toxicology*, *93*(10), 2811–2822. https://doi.org/10.1007/s00204-019-02559-7

Stýblo, M., Venkatratnam, A., Fry, R. C., & Thomas, D. J. (2021). Origins, fate, and actions of methylated trivalent metabolites of inorganic arsenic: Progress and prospects. *Archives of Toxicology*. https://doi.org/10.1007/s00204-021-03028-w

Thomas, D. J., Li, J., Waters, S. B., Xing, W., Adair, B. M., Drobna, Z., Devesa, V., & Styblo, M. (2007). Arsenic (+3 Oxidation State) Methyltransferase and the Methylation of Arsenicals. *Experimental Biology and Medicine (Maywood, N.J.)*, *232*(1), 3–13.

Thomas, D. J., Styblo, M., & Lin, S. (2001). The Cellular Metabolism and Systemic Toxicity of Arsenic. *Toxicology and Applied Pharmacology*, *176*(2), 127–144. https://doi.org/10.1006/taap.2001.9258

Tsang, V., Fry, R. C., Niculescu, M. D., Rager, J. E., Saunders, J., Paul, D. S., Zeisel, S. H., Waalkes, M. P., Stýblo, M., & Drobná, Z. (2012). The epigenetic effects of a high prenatal folate intake in male mouse fetuses exposed in utero to arsenic. *Toxicology and Applied Pharmacology*, *264*(3), 439–450. https://doi.org/10.1016/j.taap.2012.08.022

Turner, F. B., Andreassi 2nd, J. L., Ferguson, J., Titus, S., Tse, A., Taylor, S. M., & Moran, R. G. (1999). Tissue-specific expression of functional isoforms of mouse folypoly-gamma-glutamae synthetase: A basis for targeting folate antimetabolites. *Cancer Research*, *59*(24), 6074–6079.

Ussar, S., Fujisaka, S., & Kahn, C. R. (2016). Interactions between host genetics and gut microbiome in diabetes and metabolic syndrome. *Molecular Metabolism*, *5*(9), 795–803. https://doi.org/10.1016/j.molmet.2016.07.004

Vahter, M. (1999). Methylation of Inorganic Arsenic in Different Mammalian Species and Population Groups. *Science Progress*, *82*(1), 69–88. https://doi.org/10.1177/003685049908200104

Wang, D., Zhai, J.-X., & Liu, D.-W. (2017). Serum folate, vitamin B12 levels and diabetic peripheral neuropathy in type 2 diabetes: A meta-analysis. *Molecular and Cellular Endocrinology*, *443*, 72–79. https://doi.org/10.1016/j.mce.2017.01.006

Wani, N. A., Thakur, S., & Kaur, J. (2012). Mechanism of intestinal folate transport during folate deficiency in rodent model. *The Indian Journal of Medical Research*, *136*(5), 758–765.

Yang, C., Xie, L.-Y., Windle, J. J., Taylor, S. M., & Moran, R. G. (2014). Humanizing mouse folate metabolism: Conversion of the dual-promoter mouse folylpolyglutamate synthetase gene

to the human single-promoter structure. *The FASEB Journal*, *28*(5), 1998–2008. https://doi.org/10.1096/fj.13-243261

Zablotska, L. B., Chen, Y., Graziano, J. H., Parvez, F., van Geen, A., Howe, G. R., & Ahsan, H. (2008). Protective Effects of B Vitamins and Antioxidants on the Risk of Arsenic-Related Skin Lesions in Bangladesh. *Environmental Health Perspectives*, *116*(8), 1056–1062. https://doi.org/10.1289/ehp.10707

Zhang, X., Xu, X., Zhong, Y., Power, M. C., Taylor, B. D., & Carrillo, G. (2019). Serum folate levels and urinary arsenic methylation profiles in the US population: NHANES, 2003-2012. *Journal of Exposure Science & Environmental Epidemiology*, *29*(3), 323–334. https://doi.org/10.1038/s41370-018-0021-5