Published in final edited form as:

*Arch Toxicol*. 2017 July ; 91(7): 2617–2627. doi:10.1007/s00204-016-1890-9.

# Knockout of arsenic (+3 oxidation state) methyltransferase is associated with adverse metabolic phenotype in mice: The role of sex and arsenic exposure

**Christelle Douillet**[*], **Madelyn C. Huang**[†], **R. Jesse Saunders**[*], **Ellen N. Dover**[†], **Chongben Zhang**[*], and **Miroslav Stýblo**[*,†,1]

[*]Department of Nutrition, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[†]Curriculum in Toxicology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

## Abstract

Susceptibility to toxic effects of inorganic arsenic (iAs) depends, in part, on efficiency of iAs methylation by arsenic (+3 oxidation state) methyltransferase (AS3MT). *As3mt*-knockout (KO) mice that cannot efficiently methylate iAs represent an ideal model to study the association between iAs metabolism and adverse effects of iAs exposure, including effects on metabolic phenotype. The present study compared measures of glucose metabolism, insulin resistance and obesity in male and female wild-type (WT) and *As3mt*-KO mice during a 24-week exposure to iAs in drinking water (0.1 or 1 mg As/L) and in control WT and *As3mt*-KO mice drinking deionized water. Results show that effects of iAs exposure on fasting blood glucose (FBG) and glucose tolerance in either WT or KO mice were relatively minor and varied during the exposure. The major effects were associated with *As3mt* KO. Both male and female control KO mice had higher body mass with higher percentage of fat than their respective WT controls. However, only male KO mice were insulin resistant as indicated by high FBG, and high plasma insulin at fasting state and 15 minutes after glucose challenge. Exposure to iAs increased fat mass and insulin resistance in both male and female KO mice, but had no significant effects on body composition or insulin resistance in WT mice. These data suggest that *As3mt* KO is associated with an adverse metabolic phenotype that is characterized by obesity and insulin resistance, and that the extent of the impairment depends on sex and exposure to iAs, including exposure to iAs from mouse diet.

### Keywords

arsenic; metabolic phenotype; As3mt knockout mice; obesity; insulin resistance

[1]Corresponding author: Miroslav Stýblo, PhD, Department of Nutrition, CB# 7461, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7461; Phone: (919) 966-5721; Fax: (919) 843-0776; styblo@med.unc.edu.

## Introduction

Arsenic (+3 oxidation state) methyltransferase (AS3MT) is the key enzyme in the metabolism of inorganic (i) arsenic (As) (Lin et al. 2001). It catalyzes the S-adenosylmethionine-dependent methylation of iAs yielding methyl-As (MAs), dimethyl-As (DMAs) and trimethyl-As (TMAs) metabolites that contain either trivalent $As^{III}$ or pentavalent arsenic $As^{V}$ (Thomas et al. 2007). The methylation of iAs is essential for clearance of As from the body (Drobná et al. 2009), and thus plays a detoxification function. However, the methylated trivalent arsenicals ($MAs^{III}$ and $DMAs^{III}$) are more toxic and biologically active than iAs (Stýblo et al. 2000, 2002). Both population and laboratory studies show that impaired methylation of iAs is associated with an increased acute iAs toxicity or with an increased susceptibility to adverse effects associated with chronic exposure to iAs (Antonelli et al. 2014; Tseng et al. 2009). In 2009, a strain of C57BL/6 mice knocked out (KO) for *As3mt* was established by Thomas and coworkers (Drobná et al. 2009) to provide a laboratory model for *in vivo* studies examining the role of iAs methylation in adverse effects of iAs exposure. Several previous studies have described the pattern of iAs metabolism in *As3mt*-KO mice and confirmed that very little methylated arsenicals are produced after exposure to iAs (Chen et al. 2011; Drobná et al. 2009; Hughes et al. 2010). However, little is known about the susceptibility of these mice to iAs toxicity. Few studies published to date have focused mainly on effects of iAs exposure in urinary bladder of *As3mt*-KO mice (Arnold et al. 2014; Dodmane et al. 2013, 2014; Yokohira et al. 2010, 2011). We have recently compared urine and plasma metabolomes of wild-type (WT) and *As3mt*-KO mice and found that *As3mt* KO is associated with significant shifts in levels of metabolites in pathways of amino acid, carbohydrate and lipid metabolism (Huang et al. 2016). We have also found plasma triglycerides levels to be higher in male *As3mt*-KO as compared to male WT mice after exposure to iAs. Taken together, our findings suggest that *As3mt* KO has a broader impact on major metabolic pathways and may be associated with an increased susceptibility to metabolic diseases in iAs-exposed mice.

The International Agency for Research of Cancer (IARC) has classified iAs as a human carcinogen (IARC 2004). Based on the IARC review, chronic exposures to iAs in drinking water have been linked to an increased risk of skin, bladder, lung and possibly liver cancer. DNA damage by reactive oxygen species generated in response to iAs exposure and inhibition of DNA repair mechanisms by iAs or its metabolites have been suggested as potential mechanisms underlying the carcinogenic effects of iAs (Kligerman et al. 2010; Wnek et al. 2011). iAs and its trivalent methylated metabolites, $MAs^{III}$ and $DMAs^{III}$, are also potent endocrine disruptors that affect several pathways and mechanisms regulating hormone production or function, including glucose stimulated insulin secretion by pancreas (Díaz-Villaseñor et al. 2006; Douillet et al., 2013; Fu et al. 2010) or insulin signaling and insulin-dependent glucose uptake in adipocytes (Walton et al. 2004; Paul et al. 2007a). Inhibition of these mechanisms is thought to be responsible for an impaired glucose homeostasis and increased risk of diabetes that have been reported in both population studies and in laboratory studies using mice or rats exposed to iAs (Maull et al. 2012). Human exposures to iAs have also been linked to adverse metabolic phenotypes characterized by dysglycemia, dyslipidemia, inflammation and high blood pressure, the established risk

factors for diabetes and cardiovascular disease (Abhyankar et al. 2012; Chen et al. 2012; Maull et al. 2012; Moon et al. 2012; States et al. 2009; Wang et al. 2007; Wu et al. 2012). Notably, the capacity to methylate iAs (as characterized by profiles of iAs and the methylated metabolites in urine) have been consistently shown to affect the risk of cancers and cardiometabolic disease in populations chronically exposed to iAs (Ahsan et al. 2007; Chen et al. 2003; Chen et al. 2009; Del Razo et al. 2011; Huang et al. 2008; Kim et al., 2013; Li et al. 2013; Maull et al. 2012; Mendez et al., 2016; Nizam et al. 2013; Yu et al. 2000).

The goal of the present study was to examine the role of iAs methylation as a potential modulator of the effects of iAs exposure on metabolic phenotype by comparing measures of glucose metabolism, insulin resistance, and obesity in WT and *As3mt*-KO C57BL/6 mice exposed to iAs with respective unexposed controls. Results suggest that *As3mt* KO results in increased fat accumulation, insulin resistance, and impaired glucose metabolism. The extent of these effects depends on sex of mice and on the level of exposure.

## Materials and Methods

### Mice and treatment

All procedures involving mice were approved by the University of North Carolina (UNC) Institutional Animal Care and Use Committee. Male and female C57BL/6J WT mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and let to acclimatize at the UNC Animal Facilities for one week. Male and female *As3mt*-KO mice on a C57BL/6 background (Drobná, et al. 2009) were bred at the UNC Animal Facilities. Both WT and *As3mt*-KO mice were housed under controlled conditions with 12-h light/dark cycle at 22±1°C and 50±10% relative humidity (3–5 mice per cage) and with unlimited access to pelleted 2920X Teklad rodent chow (Envigo, Madison, WI, USA). All mice were 21-week old at the beginning of the study when they were randomly assigned to treatment groups with 10–14 animals per group. Both WT and *As3mt*-KO mice drank for 24 weeks (*ad libitum*) either deionized water or deionized water containing sodium arsenite ($AsNaO_2$, ≥99% pure; Sigma-Aldrich, St. Louis, MO, USA) at final concentrations of 0.1 or 1.0 mg As/L (i.e., 0.1 or 1 ppm). Water with sodium arsenite was prepared weekly to minimize oxidation of $iAs^{III}$ to $iAs^{V}$. Food and water consumption and body weight were monitored in all treatment groups weekly.

### Metabolic phenotyping

The study design is summarized in Supplemental Figure 1. Fasting blood glucose (FBG) was measured at week 1, 8, 12 and 24 of iAs exposure. Mice were fasted for 6 hours and FBG levels were measured in blood collected from tail cuts using One Touch Ultra 2 glucometer (LifeScan, Milpitas, CA, USA). In addition to measurements of FBG, a glucose tolerance test was administered to all mice at week 1 and 8. Here, fasted mice were injected i.p. with 2 g/kg b.w. of D-glucose (Sigma-Aldrich) dissolved in Dulbecco's phosphate buffered saline (Mediatech, Manassas, VA, USA) and glucose levels were measured in blood collected by tail bleeds 15, 30, 60 and 120 minutes post-injection. To quantify glucose tolerance, area under the curve (AUC) was determined for a plot of blood glucose (mg/dL) vs. time (min)

using Prism 5 (GraphPad, La Jolla, CA, USA). To examine insulin resistance, plasma insulin was measured in fasted mice (week 12 and 24) and 15 minutes after i.p. injection of D-glucose (2 g/kg b.w.) (week 12); plasma was isolated from blood collected by tail bleeds. The levels of insulin in plasma were determined by ELISA (EMD Millipore, St Charles, MO, USA) following the manufacturer's protocol. Body composition (lean and fat mass) was determined at week 19 by magnetic resonance imaging (MRI) using EchoMRI 3-in-1 Composition Analyzer and Labmaster v.3.2.2 software (Echo Medical Systems, Houston, TX, USA).

### Sample collection at sacrifice

After 24 weeks, all mice were fasted for 6 hours and spot urine samples were collected by placing a glass capillary tube next to the urethral meatus and applying gentle abdominal pressure. Mice were then sacrificed by cervical dislocation. Half of the mice in each treatment group were injected i.p. with insulin (2U/kg b.w.) exactly 10 minutes prior to sacrifice. Samples of liver (right medial lobes) were collected during the dissection, snapped frozen in liquid nitrogen, and stored along with urine samples at −80°C.

### Assessment of insulin-activated signal transduction in the liver

The levels of total and phosphorylated protein kinase B (Akt) were determined in livers of mice injected with insulin prior to sacrifice as previously described (Paul et al. 2007a). Briefly, liver tissue was homogenized in a lysis buffer containing protease and phosphatase inhibitors. The homogenate was sonicated for 10 seconds and then centrifugated at 16,000g for 10 min at 4°C. The protein concentration of the supernatants was determined by Bicinchoninic Assay (Pierce Thermofisher, Waltham, MA, USA). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes for immunoblot analysis. The phosphorylated Akt (p-Akt/Ser 473) and total Akt were detected using monoclonal antibodies from Cell Signaling (Danvers, MA, USA). The immunoblots were quantified using a Versadoc imaging system (Biorad, Hercules, CA, USA). Each blot contained one liver supernate from each treatment group. One unique liver supernate was used as an internal standard on every immunoblot. The ratio of p-Akt/Akt was calculated for each liver supernate and expressed as a percentage of the p-Akt/Akt ratio of the internal standard for the given blot. This approach controlled for blot-to-blot variations and allowed comparison of the p-Akt/Akt values across the treatment groups.

### Speciation analysis of arsenic

Arsenic species in samples of urine and in phosphoric acid-digested liver homogenates were measured by hydride generation-atomic absorption spectrometry coupled with a cryotrap (HG-CT-AAS) as previously described (Hernández-Zavala et al. 2008; Currier et al. 2011). Calibration curves for quantification of As species were generated using aqueous solutions of pentavalent As standards, including sodium arsenate (≥99% pure) from Sigma-Aldrich, and methylarsonic and dimethylarsinic acids (98% pure) from Chem Service (West Chester, PA, USA). All samples and standards were treated with 2% cysteine for 60 minutes prior to analysis to convert all pentavalent arsenicals to trivalency (Matoušek et al. 2008). As applied in this study, the HG-CT-AAS analysis determined concentrations of total iAs (i.e., $iAs^{III}$ + $iAs^{V}$), total MAs ($MAs^{III}$ + $MAs^{V}$) and total DMAs ($DMAs^{III}$ + $DMAs^{V}$); no TMAs was

detected in either urines or livers. HG-CT-AAS was also used to determine the content and speciation of As in 3 lots of the 2920X Teklad mouse diet that were fed to the mice during the 24 weeks of the study. Here, 4–6 pellets from each lot were powdered and microwave-digested in ultrapure phosphoric acid (J. T. Baker, Phillipsburg, NJ, USA); the digestates were treated with 2% cysteine before analysis.

### Statistical analysis

ANOVA with Fisher's least significant differences posttest for multiple comparisons were used to evaluate differences between treatment groups in the measures of glucose and insulin metabolism, in p-Akt/Akt ratio, in body composition and mass, and in the concentrations of total As and As species in urines and livers. Data were expressed as mean ± standard error (SE); *P*-values less than 0.05 were considered statistically significant. All statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

## Results

### Food and water consumption, and body mass

Exposure to iAs had no significant effects on the amount of food or water consumed by either WT or *As3mt*-KO mice (Suppl. Figure 2). However, male WT mice consumed significantly more food and water than WT females or male or female *As3mt*-KO mice ($p<0.05$). There were no significant differences in either food or water consumption between male and female KO mice. The *As3mt*-KO mice, males and females, had significantly greater body mass than their WT counterparts already at the beginning of the study ($p<0.0001$) and this difference remained statistically significant at week 24 ($p<0.0001$) (Suppl. Figure 3). In addition, as a result of the random assignment to the treatment groups, male and female KO mice in the 0.1 ppm group were heavier than their KO counterparts in the control and 1 ppm groups at the start of the exposure ($p\leq0.013$), and remained heavier throughout the study.

All mice gained weight between week 0 and week 24 (Suppl. Figure 4), but the average absolute and relative weight gains differed between the treatment groups. Control male WT mice gained more weight than control WT females. Exposure to 1 ppm iAs decreased the weight gain in WT males and increased it in WT as well as KO females. Female KO mice in control and 1 ppm groups gained more weight than WT females in the respective treatment groups. In contrast, exposure to iAs had no significant effects on weight gain of male KO mice. There were no significant differences in weight gains between KO males and KO females regardless of treatment.

### Metabolic phenotypes

Phenotyping was carried out throughout the study. The time points for individual tests were selected to reduce stress associated with animal handling and with invasive procedures involving injections and blood collections, and also to allow enough time for healing and recovery.

**Fasting glycemia—**FBG was measured at 3 time points during the study (week 1, 8, and 12) and at sacrifice (week 24). In general, control and iAs-treated male *As3mt*-KO mice had the highest FBG levels (Figure 1), although the differences between this and the other groups were not always statistically significant. The extent and directions of the effects of iAs exposure on FBG varied throughout the study. At week 1, male WT and male KO mice exposed to 0.1 and 1 ppm As had higher FBG than the respective controls, but there were no statistically significant differences in FPG of iAs-treated and control WT or KO females. In contrast, male KO and female WT mice exposed to 0.1 ppm As for 8 weeks had lower FBG than mice in the respective control groups and in groups exposed to 1 ppm As; iAs exposure had little effects on FPG in male WT and female KO mice. At week 12, the only group affected by iAs exposure were male WT mice drinking water with 0.1 ppm As – FBG was significantly higher in this group as compared to the male WT control and 1 ppm groups. No statistically significant effects of iAs exposure were found at week 24.

**Glucose tolerance—**An i.p. glucose tolerance test (IP-GTT) was administered at week 1 and 8; AUC was calculated for each mouse. At week 1, male *As3mt*-KO mice were less glucose tolerant than male and female mice in other groups as indicated by higher AUC values (Figure 2). iAs exposure had no significant effects on AUC in male WT and KO or in female WT mice; however, exposure to 0.1 ppm As increased AUC in female KO mice. There were little or no differences in AUC values between WT and to *As3mt* KO mice at week 8. At this time point, male WT and male KO mice were less glucose tolerant than female mice in the respective groups. Exposure to iAs increased AUC in male KO mice, but only at 0.1 ppm level, and had no effects on AUC in other groups.

**Insulin resistance—**Fasting plasma insulin (FPI) levels determined at week 12 were significantly higher in male KO mice as compared to KO females and to WT males or females (Figure 3a). There were no significant differences in FPI between male and female WT and female KO mice regardless of treatment; the difference between KO females in the control and 0.1 ppm groups was only marginally significant ($p=0.052$). Male KO mice exposed to 0.1 ppm As had significantly higher FPI than KO males in the control and 1 ppm groups. FPI levels in female KO mice were significantly lower than those in male KO mice. Results of FPI analysis at week 24 were generally consistent with those at week 12; however, here exposure to 0.1 ppm increased FPI in both male and female KO mice. The response of pancreas to glucose challenge was tested only at week 12. The blood glucose levels 15 minutes after i.p. injection of glucose were consistently lower in female KO as compared to female WT mice, regardless of treatment (Figure 4a). Male KO mice exposed to 0.1 ppm As had higher 15-min blood glucose than control male KO. The differences in 15-min plasma insulin resembled those found in fasting plasma – insulin concentrations were higher in plasma of male and female KO mice as compared to WT males and females, respectively (Figure 4b). There were also differences between KO male and KO female mice – insulin levels were significantly higher in the male groups. No statistically significant effects of iAs exposure were found; the lowest p value of 0.071 was found for the difference between plasma insulin levels of control KO female mice and KO females exposed to 0.1 ppm As.

To further characterize insulin resistance, we measured total Akt and p-Akt in livers of mice injected with insulin prior to sacrifice at week 24 (Suppl. Figure 5). The highest p-Akt/Akt ratios were found in livers of WT female mice and the lowest in livers of KO males. Because of substantial variations, very few differences between the treatment groups were statistically significant. However, the average p-Akt/Akt ratio correlated negatively with average FPI level measured at sacrifice: $R^2$=0.51, $p < 0.01$ (Figure 5).

**Body composition—**The MRI analysis carried out at week 19 showed that fat and lean mass differed significantly between WT and KO mice – the bodies of male and female KO mice contained significantly more fat that those of male and female WT mice (Figure 6a). In addition, male KO mice had less lean mass than WT males. Fat represented 31% and 27% of body mass of control male and female KO mice, respectively, as compared to 11% for control male and 12.5% for control female WT mice (Figure 6b). Male KO mice exposed to 0.1 ppm As had more fat than males in the KO control or 1ppm group, but %fat was not significantly different. Female KO mice exposed to either 0.1 or 1 ppm had more fat and %fat than KO control females. iAs exposure had no significant effects on fat mass or %fat of WT male and female mice.

## Arsenic species in urine and liver

Urine and livers for As analysis were collected at sacrifice (week 24). Total As concentration in urine (iAs + MAs + DMAs) of both WT and KO mice increased with the level of iAs exposure in a dose-dependent manner (Figure 7a). DMAs was the major As species in urines of WT mice, followed by iAs and MAs. Urines of KO mice contained almost exclusively iAs; only traces of MAs and DMAs were detected. Total As levels were significantly lower in urine of male KO mice exposed to 1 ppm As than in urine of male WT mice at the same exposure level; no other differences due to KO or sex were found within the exposure groups. More pronounced differences were found in As content of the livers (Figure 7b). *As3mt* KO was associated with significantly higher levels of total As in all treatment groups. Sex was also an important factor – livers of female KO mice exposed to 0.1 or 1 ppm As contained more total As than livers of the respective male KO mice. In contrast, there were no significant differences in total As levels between WT male and female mice; no significant effects of iAs exposure were found. Similar to urine, livers of KO mice contained almost exclusively iAs. Notably, urine and livers of control WT and KO mice that drank only deionized water contained relatively high levels of As, suggesting that these mice were exposed to As from diet.

## Arsenic concentration in the diet

To determine if diet was an additional source of As exposure, we analyzed three lots of 2920X Teklad chow that were used in the course of the study. The concentrations of As in these lots ranged from 110 to 136 μg As/kg (i.e., 0.11 and 0.136 ppm); iAs was the only As species found with traces of MAs detected in one of the lots.

## Discussion

Environmental exposures to iAs have been shown to increase the risk of metabolic disease, including diabetes and cardiovascular disease (Abhyankar et al. 2012; Chen et al. 2012; Maull et al. 2012; Moon et al. 2012; States et al. 2009; Wang et al. 2007; Wu et al. 2012). However, with only a limited number of laboratory studies carried out to date, mechanisms underlying the metabolic effects of iAs are poorly understood. Overwhelming evidence suggests that the capacity to methylate iAs plays a major role in susceptibility to these effects (Ahsan et al. 2007; Chen et al. 2003; Chen et al. 2009; Del Razo et al. 2011; Huang et al. 2008; Kim et al., 2013; Li et al. 2013; Maull et al. 2012; Mendez et al., 2016; Nizam et al. 2013; Yu et al. 2000). The *As3mt*-KO mouse strain was created to facilitate laboratory research focusing on the role of iAs methylation in the adverse effects of iAs exposure. Basic characteristics of iAs metabolism in *As3mt*-KO mice have been previously published. These mice are not able to effectively methylate iAs and retain large amounts of iAs in tissues; the clearance of iAs from the body is slow when compared to WT mice (Drobná et al. 2009; Hughes et al. 2008). Little is known about other metabolic pathways that could be affected by *As3mt* KO. Although the only known function of As3mt is the methylation of iAs, it is possible that this enzyme is involved in other methylation reactions. We have recently compared global metabolomics profiles of *As3mt*-KO and WT mice exposed to iAs and of the unexposed WT and KO controls (Huang et al. 2016). We found significant differences in both plasma and urinary metabolomes. These differences were sex-dependent and pointed to an altered carbohydrate and lipid metabolism, suggesting that *As3mt*-KO mice may be more susceptible to developing an adverse metabolic phenotype. The goal of the present study was to investigate and characterize this phenotype.

We have previously reported that exposure of male WT C57BL/6 mice to 50 ppm As (as arsenite) in drinking water, alone (Paul et al. 2007b) or combined with high-fat diet (Paul et al. 2011), resulted in impaired glucose tolerance and in a failure of pancreas to secrete insulin in response to glucose challenge. In contrast, the present study using much lower levels of iAs exposure (0.1 and 1 ppm) found only minor effects on FBG or glucose tolerance in either WT and KO mice, and these effects varied over the time of the study (Figures 1 and 2). The lack of a more pronounced effect on glucose metabolism after exposure to 0.1 and 1 ppm suggests that mice, that metabolize iAs more efficiently than humans (Vahter 1994), may have to be exposed to higher levels of iAs to reproduce the metabolic effects of iAs reported in population studies. It is also possible that the mice were adapted to iAs because they were exposed to relatively high levels of iAs in the diet prior to (and during) the exposure to iAs in drinking water. In fact, the differences in metabolic phenotypes of WT and KO mice in the control groups (not exposed to iAs in drinking water) could be, at least in part, due to the exposure to iAs from the diet and due to the limited capacity of KO mice to metabolize this iAs, resulting in its accumulation in the metabolically active tissues, including liver (Figures 7). Notably, exposure to As from diet has not been examined or taken into account in most published studies on the effects of iAs exposure in laboratory rodents, thus confusing the reported results and their interpretation. Results of our study highlight the need to monitor and account for the background exposure

to iAs from animal diets used in laboratory studies, especially in studies examining effects of very low (ppb levels) exposures to iAs from other sources, including drinking water.

In spite of the variations, male KO mice, both control and iAs-treated, appeared to have higher FBG and higher AUC values than males and females in other treatment groups (Figures 1 and 2). Male KO mice had also significantly higher FPI and plasma insulin 15 minutes after glucose challenge (Figures 3 and 4). Taken together, these findings are indicative of insulin resistance. Insulin resistance is usually associated with obesity and can progress to type-2 diabetes if followed by β-cell dysfunction and failure of β-cells to secrete sufficient amounts of insulin in response to high blood glucose (Ferrannini 1998; Weyer et al. 2001). In this study male KO mice accumulated more fat and had higher % body fat than either WT males or WT females (Figure 6). Notably, KO males became obese while consuming less food than lean WT males (Suppl. Figure 2b), suggesting a major difference in energy expenditure. The KO female mice accumulated almost as much fat as KO males and there was no difference in % body fat among these two groups. However, despite being as obese as KO males and having higher levels of As in the liver (Figure 7), the KO females were not as insulin resistant as indicated by significantly lower FPI and 15-minute plasma insulin levels (Figures 3 and 4). Thus, the fat accumulation or iAs disposition alone cannot explain the differences in insulin resistance between KO male and KO female mice. It is possible that *As3mt* KO affects in a sex-dependent manner expression of genes regulating carbohydrate, lipid and/or energy metabolism, and that a modified expression of these genes is responsible for or contributes to the adverse metabolic phenotype of KO mice. A comprehensive analysis of transcriptomes of male and female WT and *As3mt*-KO mice, as well as comparison of energy metabolism and expenditure between the two mouse strains and the sexes would help to answer this question.

The exposure to iAs in drinking water, particularly exposure to 0.1 ppm As, appeared to increase fat mass or %fat, and FPI in KO male and female mice, but had no effects on fat accumulation or plasma insulin in WT mice (Figures 3, 4 and 6). In general, the effects of iAs exposure were minor as compared to the major effects of *As3mt* KO and sex. These findings are consistent with results of our metabolomics study that found sex to play a major role in the metabolomic responses to *As3mt* KO in C57BL/6 mice (Huang et al. 2016). The KO-related shifts in plasma and urinary metabolomes differed significantly between males and females, while iAs exposure in drinking water (1 ppm) had only minor effects. Metabolism of lipids, specifically phosphatidylcholines, was among the most affected pathways in *As3mt*-KO mice (Huang et al. in press). Notably, phosphatidylcholines make up 60–80% of the phospholipid component of plasma lipoproteins (Cole et al. 2012) and dysregulation of plasma lipoproteins, including low density lipoproteins (LDL) and high-density lipoproteins (HDL), has been directly linked to diabetes, obesity, and metabolic disease in general (Chan et al. 2004).

In summary, our data show that *As3mt* KO in mice resulted in an adverse phenotype that was characterized primarily by obesity and insulin resistance. However, insulin resistance was much more pronounced in male KO as compared to female KO mice. The exposure to 0.1 and 1 ppm As (as arsenite) in drinking water further exacerbated this phenotype in both male and female KO mice, but the effects were not always statistically significant. The

background exposure to relatively high levels of iAs in the diet or effects of *As3mt* KO on expression of metabolic genes may be responsible for the differences in the metabolic phenotypes of WT and KO mice and/or for the relatively minor responses to the additional exposure to iAs from drinking water. The mechanisms by which *As3mt* KO alters metabolic phenotype in a sex-dependent manner in the absence or presence of iAs exposure are unclear and should be investigated in future studies. These studies should control for As levels in laboratory diet and should examine effects of iAs exposure in the glucose utilizing tissues and in the pancreas, including effects of *As3mt* KO on expression of genes regulating major metabolic pathways and energy metabolism.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

**Funding**

This work was supported by grants from the National Institute of Health (R01ES022697 and DK 056350), and in part by National Research Service Award from the National Institute of Environmental Health Sciences, NIH (T32 ES007126).

The authors thank Dr. David Thomas (US EPA) for his continuous support and advice regarding the establishment and maintenance of *As3mt*-KO mouse colony at UNC Chapel Hill.

## References

Abhyankar LN, Jones MR, Guallar E, Navas-Acien A. Arsenic exposure and hypertension: a systematic review. Environ Health Perspect. 2012; 120:494–500. [PubMed: 22138666]

Ahsan H, Chen Y, Kibriya MG, Slavkovich V, Parvez F, Jasmine F, Gamble MV, Graziano JH. Arsenic metabolism, genetic susceptibility, and risk of premalignant skin lesions in Bangladesh. Cancer Epidemiol Biomarkers Prev. 2007; 16:1270–1278. [PubMed: 17548696]

Antonelli R, Shao K, Thomas DJ, Sams R II, Cowden J. AS3MT, GSTO, and PNP polymorphisms: Impact on arsenic methylation and implications for disease susceptibility. Environ Res. 2014; 132:156–167. [PubMed: 24792412]

Arnold LL, Suzuki S, Yokohira M, Kakiuchi-Kiyota S, Pennington KL, Cohen SM. Time course of urothelial changes in rats and mice orally administered arsenite. Toxicol Pathol. 2014; 42:855–862. [PubMed: 23690446]

Chan DC, Barrett PH, Watts GF. Lipoprotein transport in the metabolic syndrome: pathophysiological and interventional studies employing stable isotopy and modelling methods. Clin Sci (Lond). 2004; 107:233–249. [PubMed: 15225143]

Chen YC, Guo YL, Su HJ, Hsueh YM, Smith TJ, Ryan LM, Lee MS, Chao SC, Lee JY, Christiani DC. Arsenic methylation and skin cancer risk in southwestern Taiwan. J Occup Environ Med. 2003; 45:241–248. [PubMed: 12661181]

Chen Y, Parvez F, Gamble M, Islam T, Ahmed A, Argos M, Graziano JH, Ahsan H. Arsenic exposure at low-to-moderate levels and skin lesions, arsenic metabolism, neurological functions, and biomarkers for respiratory and cardiovascular diseases: review of recent findings from the Health Effects of Arsenic Longitudinal Study (HEALS) in Bangladesh. Toxicol Appl Pharmacol. 2009; 239:184–192. [PubMed: 19371619]

Chen B, Arnold LL, Cohen SM, Thomas DJ, Le XC. Mouse arsenic (+3 oxidation state) methyltransferase genotype affects metabolism and tissue dosimetry of arsenicals after arsenite administration in drinking water. Toxicol Sci. 2011; 124:320–326. [PubMed: 21934131]

Chen JW, Wang SL, Wang YH, Sun CW, Huang YL, Chen CJ, et al. Arsenic methylation, GSTO1 polymorphisms, and metabolic syndrome in an arseniasis endemic area of southwestern Taiwan. Chemosphere. 2012; 88:432–438. [PubMed: 22440634]

Cole LK, Vance JE, Vance DE. Phosphatidylcholine biosynthesis and lipoprotein metabolism. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 2012; 1821:754–761. [PubMed: 21979151]

Currier JM, Svoboda M, Matoušek T, Dědina J, Stýblo M. Direct analysis and stability of methylated trivalent arsenic metabolites in cells and tissues. Metallomics. 2011; 3:1347–1354. [PubMed: 22015847]

Del Razo LM, García-Vargas GG, Valenzuela OL, Hernandez-Castellanos E, Sánchez-Peña LC, Drobná Z, Loomis D, Stýblo M. Exposure to arsenic in drinking water is associated with increased prevalence of diabetes: a cross-sectional study in the Zimapán and Lagunera Regions in Mexico. Environ Health. 2011; 10:73. [PubMed: 21864395]

Díaz-Villaseñor A, Sánchez-Soto MC, Cebrián ME, Os-trosky-Wegman P, Hiriart M. Sodium arsenite impairs insulin secretion and transcription in pancreatic β-cells. Toxicol Appl Pharmacol. 2006; 214:30–34. [PubMed: 16413591]

Dodmane PR, Arnold LL, Pennington KL, Thomas DJ, Cohen SM. Effect of dietary treatment with dimethylarsinous acid (DMA(III)) on the urinary bladder epithelium of arsenic (+3 oxidation state) methyltransferase (As3mt) knockout and C57BL/6 wild type female mice. Toxicology. 2013; 305:130–135. [PubMed: 23376817]

Dodmane PR, Arnold LL, Muirhead DE, Suzuki S, Yokohira M, Pennington KL, Dave BJ, Lu X, Le XC, Cohen SM. Characterization of intracellular inclusions in the urothelium of mice exposed to inorganic arsenic. Toxicol Sci. 2014; 137:36–46. [PubMed: 24097667]

Douillet C, Currier JM, Saunders J, Bodnar W, Matoušek T, Stýblo M. Methylated trivalent arsenicals are potent inhibitors of glucose stimulated insulin secretion by murine pancreatic islets. Toxicol Appl Pharmacol. 2013; 267:11–15. [PubMed: 23261974]

Drobná Z, Naranmandura H, Kubachka KM, Edwards BC, Herbin-Davis K, Styblo M, Le XC, Creed JT, Maeda N, Hughes MF, et al. 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. Chem Res Toxicol. 2009; 22:1713–1720. [PubMed: 19691357]

Ferrannini E. Insulin resistance versus insulin deficiency in non-insulindependent diabetes mellitus: problems and prospects. Endocr Rev. 1998; 19:477–490. [PubMed: 9715376]

Fu J, Woods CG, Yehuda-Shnaidman E, Zhang Q, Wong V, Collins S, Sun G, Andersen ME, Pi J. Low-Level Arsenic Impairs Glucose-Stimulated Insulin Secretion in Pancreatic Beta Cells: Involvement of Cellular Adaptive Response to Oxidative Stress. Environ Health Perspect. 2010; 118:864–870. [PubMed: 20100676]

Hernández-Zavala A, Matoušek T, Drobná Z, Adair BM, Dědina J, Thomas DJ, Stýblo M. Speciation of arsenic in biological matrices by automated hydride generation-cryotrapping-atomic absorption spectrometry with multiple microflame quartz tube atomizer (multiatomizer). J Anal At Spectrom. 2008; 23:342–351. [PubMed: 18677417]

Huang YK, Pu YS, Chung CJ, Shiue HS, Yang MH, Chen CJ, Hsueh YM. Plasma folate level, urinary arsenic methylation profiles, and urothelial carcinoma susceptibility. Food Chem Toxicol. 2008; 46:929–938. [PubMed: 18054417]

Huang MC, Douillet C, Su M, Zhou K, Wu T, Chen W, Galanko JA, Drobná Z, Saunders RJ, Martin E, Fry RC, Jia W, Stýblo M. Metabolomic profiles of arsenic (+3 oxidation state) methyltransferase knockout mice: Effect of sex and arsenic exposure. Arch Toxicol. 2016; in press. doi: 10.1007/s00204-016-1676-0

Huang MC, Douillet CC, Stýblo M. Knockout of arsenic (+3 oxidation state) methyltransferase results in sex-dependent changes in phosphatidylcholine metabolism in mice. Arch Toxicol. in press.

Hughes MF, Edwards BC, Herbin-Davis KM, Saunders J, Styblo M, Thomas DJ. Arsenic (+3 oxidation state) methyltransferase genotype affects steady-state distribution and clearance of arsenic in arsenate-treated mice. Toxicol Appl Pharmacol. 2010; 249:217–223. [PubMed: 20887743]

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Drinking-water Disinfectants and Contaminants, including Arsenic. Vol. 84. WHO-International Agency for Research on Cancer; Lyon, France: 2004.

Kim NH, Mason CC, Nelson RG, Afton SE, Essader AS, Medlin JE, Levine KE, Hoppin JA, Lin C, Knowler WC, Sandler DP. Arsenic exposure and incidence of type 2 diabetes in Southwestern American Indians. Am J Epidemiol. 2013; 177:962–269. [PubMed: 23504692]

Kligerman AD, Malik SI, Campbell JA. Cytogenetic insights into DNA damage and repair of lesions induced by a monomethylated trivalent arsenical. Mutat Res. 2010; 695:2–8. [PubMed: 19800024]

Li X, Li B, Xi S, Zheng Q, Wang D, Sun G. Association of urinary monomethylated arsenic concentration and risk of hypertension: a cross-sectional study from arsenic contaminated areas in northwestern China. Environ Health. 2013; 12:37. [PubMed: 23602086]

Lin S, Shi Q, Nix FB, Styblo M, Beck MA, Herbin-Davis KM, Hall LL, Simeonsson JB, Thomas DJ. A novel S-adenosyl-L-methionine:arsenic(III) methyltransferase from rat liver cytosol. J Biol Chem. 2001; 277:10795–10803.

Matoušek T, Hernández-Zavala A, Svoboda M, Langerová L, Adair BM, Drobná Z, Thomas DJ, Stýblo M, Dědina J. Oxidation state specific generation of arsines from methylated arsenicals based on L- cysteine treatment in buffered media for speciation analysis by hydride generation - automated cryotrapping - gas chromatography- atomic absorption spectrometry with the multiatomizer. Spetrochim Acta Part B. 2008; 63:396–406.

Mendez MA, González-Horta C, Sánchez-Ramírez B, Ballinas-Casarrubias L, Hernández Cerón R, Viniegra Morales D, Baeza Terrazas FA, Ishida MC, Gutiérrez-Torres DS, Saunders RJ, Drobná Z, Fry RC, Buse JB, Loomis D, García-Vargas GG, Del Razo LM, Stýblo M. Chronic exposure to arsenic and markers of cardiometabolic risk: A cross-sectional study in Chihuahua, Mexico. Environ Health Perspect. 2016; 124:104–111. [PubMed: 26068977]

Maull EA, Ahsan H, Cooper G, Edwards J, Longnecker M, Navas-Acien A, Pi J, Silbergeld E, Styblo M, Tseng CH, Thayer K, Loomis D. Evaluation of the Association be-tween Arsenic and Diabetes: A National Toxicology Pro-gram Workshop Review. Environ Health Perspect. 2012; 120:1658–1670. [PubMed: 22889723]

Moon K, Guallar E, Navas-Acien A. Arsenic exposure and cardiovascular disease: an updated systematic review. Curr Atheroscler Rep. 2012; 14:542–555. [PubMed: 22968315]

Nizam S, Kato M, Yatsuya H, Khalequzzaman M, Ohnuma S, Naito H, Nakajima T. Differences in urinary arsenic metabolites between diabetic and non-diabetic subjects in Bangladesh. Int J Environ Res Public Health. 2013; 10:1006–1019. [PubMed: 23481591]

Paul DS, Harmon AW, Devesa V, Thomas DJ, Stýblo M. Molecular mechanisms of the diabetogenic effects of arsenic: inhibition of insulin signaling by arsenite and methylarsonous acid. Environ Health Perspect. 2007a; 115:734–742. [PubMed: 17520061]

Paul DS, Hernández-Zavala A, Walton FS, Adair BM, Dědina J, Matoušek T, Styblo M. Examination of the effects of arsenic on glucose homeostasis in cell culture and animal studies: Development of a mouse model for arsenic-induced diabetes. Toxicol Appl Pharmacol. 2007b; 222:305–314. [PubMed: 17336358]

Paul DS, Walton FS, Saunders RJ, Styblo M. Characterization of the impaired glucose homeostasis produced in C57BL/6 mice by chronic exposure to arsenic and high-fat diet. Environ Health Perspect. 2011; 119:1104–1109. [PubMed: 21592922]

States JC, Srivastava S, Chen Y, Barchowsky A. Arsenic and cardiovascular disease. Toxicol Sci. 2009; 107:312–323. [PubMed: 19015167]

Stýblo M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR, Thomas DJ. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in human cells. Arch Toxicol. 2000; 74:289–299. [PubMed: 11005674]

Stýblo M, Drobná Z, Jaspers I, Lin S, Thomas DJ. The role of biomethylation in toxicity and carcinogenicity of arsenic: a research update. Environ Health Perspect. 2002; 110(Suppl 5):767–771. [PubMed: 12426129]

Thomas DJ, Li J, Waters SB, Xing W, Adair BM, Drobna Z, Devesa V, Styblo M. Arsenic (+3 oxidation state) methyltransferase and the methylation of arsenicals. Exp Biol Med (Maywood). 2007; 232:3–13. [PubMed: 17202581]

Tseng CH. A review on environmental factors regulating arsenic methylation in humans. Toxicol Appl Pharmacol. 2009; 235:338–350. [PubMed: 19168087]

Wnek SM, Kuhlman CL, Camarillo JM, Medeiros MK, Liu KJ, Lau SS, Gandolfi AJ. Interdependent genotoxic mechanisms of monomethylarsonous acid: role of ROS-induced DNA damage and poly(ADP-ribose) polymerase-1 inhibition in the malignant transformation of urothelial cells. Toxicol Appl Pharmacol. 2011; 257:1–13. [PubMed: 21925530]

Vahter M. Species Differences in the metabolism of ar-senic compounds. Appl Organomet Chem. 1994; 8:175–182.

Walton FS, Harmon AW, Paul DS, Drobna Z, Patel YM, Styblo M. Inhibition of insulin-dependent glucose uptake by trivalent arsenicals: Possible mechanism of arsenic-induced diabetes. Toxicol Appl Pharmacol. 2004; 198:424–433. Inhibition of insulin-dependent glucose uptake by trivalent arsenicals: possible mechanism of arsenic-induced diabetes. Toxicol. Appl. Pharmacol. 198:424–433. [PubMed: 15276423]

Wang SL, Chang FH, Liou SH, Wang HJ, Li WF, Hsieh DP. Inorganic arsenic exposure and its relation to metabolic syndrome in an industrial area of Taiwan. Environ Int. 2007; 33:805–811. [PubMed: 17481731]

Weyer C, Tataranni PA, Bogardus C, Pratley RE. Insulin resistance and insulin secretory dysfunction are independent predictors of worsening of glucose tolerance during each stage of type 2 diabetes development. Diabetes Care. 2001; 24:89–94. [PubMed: 11194248]

Wu F, Jasmine F, Kibriya MG, Liu M, Wójcik O, Parvez F, Rahaman R, Roy S, Paul-Brutus R, Segers S, Slavkovich V, Islam T, Levy D, Mey JL, van Geen A, Graziano JH, Ahsan H, Chen Y. Association between arsenic exposure from drinking water and plasma levels of cardiovascular markers. Am J Epidemiol. 2012; 175:1252–1261. [PubMed: 22534204]

Yokohira M, Arnold LL, Pennington KL, Suzuki S, Kakiuchi-Kiyota S, Herbin-Davis K, Thomas DJ, Cohen SM. Severe systemic toxicity and urinary bladder cytotoxicity and regenerative hyperplasia induced by arsenite in arsenic (+3 oxidation state) methyltransferase knockout mice. A preliminary report Toxicol Appl Pharmacol. 2010; 246:1–7. [PubMed: 20423714]

Yokohira M, Arnold LL, Pennington KL, Suzuki S, Kakiuchi-Kiyota S, Herbin-Davis K, Thomas DJ, Cohen SM. Effect of sodium arsenite dose administered in the drinking water on the urinary bladder epithelium of female arsenic (+3 oxidation state) methyltransferase knockout mice. Toxicol Sci. 2011; 121:257–266. [PubMed: 21385732]

Yu RC, Hsu KH, Chen CJ, Froines JR. Arsenic methylation capacity and skin cancer. Cancer Epidemiol Biomarkers Prev. 2000; 9:1259–1262. [PubMed: 11097236]

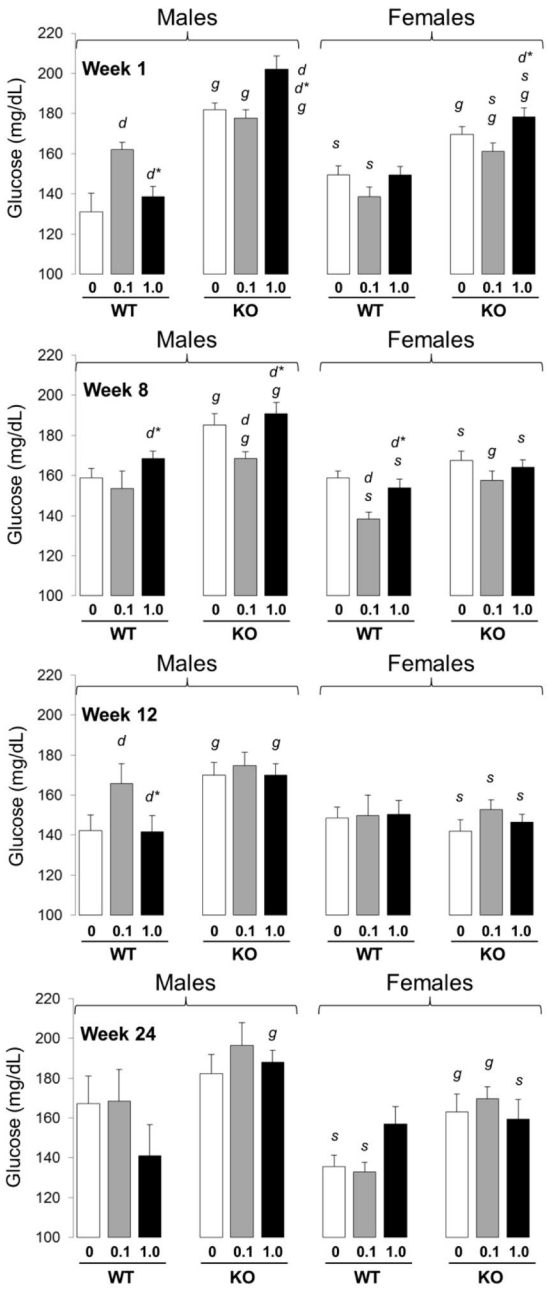


**Figure 1.**

Fasting blood glucose in male and female wild-type (WT) and *As3mt*-knockout (KO) mice after 1, 8, 12 and 24 weeks of exposure to 0, 0.1, or 1 ppm As in drinking water (Mean +SE, N = 10–14). P<0.05 for the following comparisons: *d*, 0.1 vs 0 ppm or 1 vs 0 ppm for mice of the same sex and genotype; *d**, 1 vs. 0.1 ppm for mice of the same sex and genotype; *g*, KO vs WT mice of the same sex and at the same exposure level; *s*, females vs males of the same genotype and at the same exposure level.

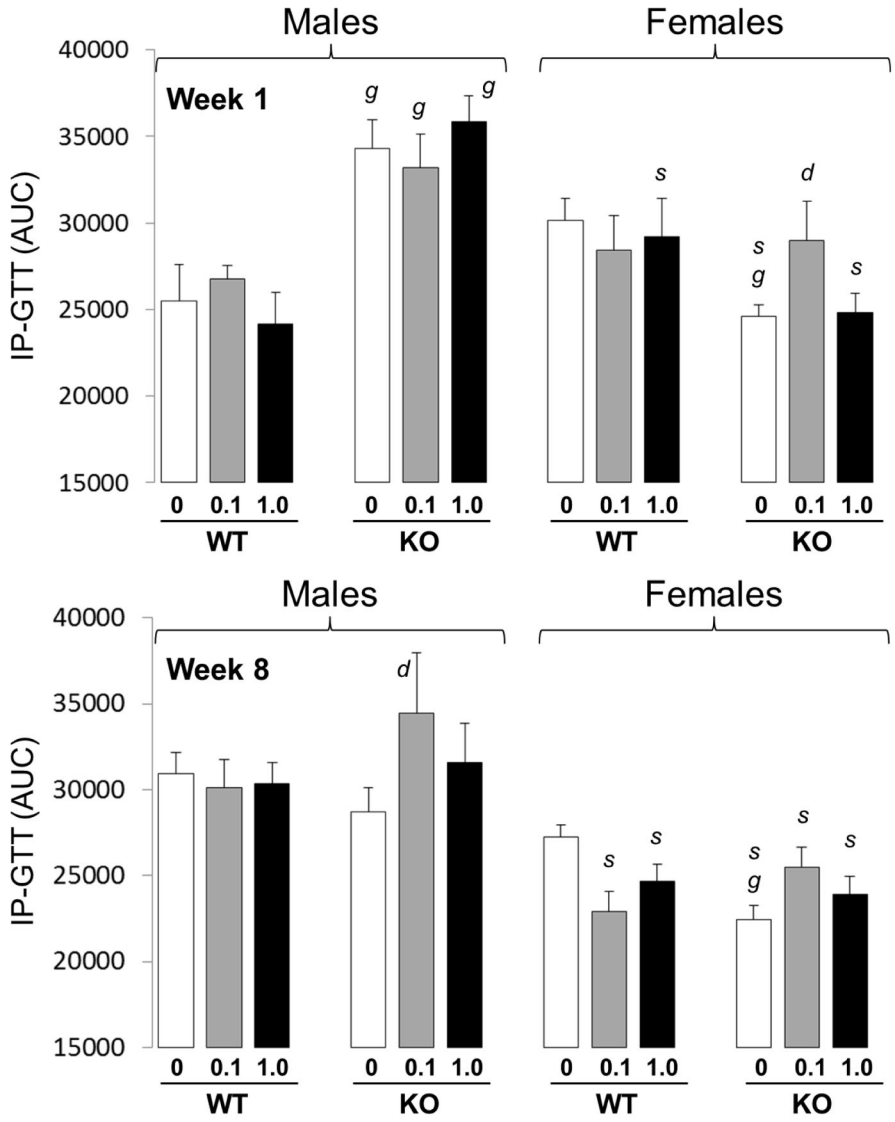


**Figure 2.**

I.p. glucose tolerance test (IP-GTT) in male and female wild-type (WT) and *As3mt*-knockout (KO) mice after 1 and 8 weeks of exposure to 0, 0.1, or 1 ppm As in drinking water. Area under the curve (AUC) was calculated for a plot of blood glucose vs time (Mean +SE, N = 10–14). P<0.05 for the following comparisons: *d*, 0.1 vs 0 ppm or 1 vs 0 ppm for mice of the same sex and genotype; *g*, KO vs WT mice of the same sex and at the same exposure level; *s*, females vs males of the same genotype and at the same exposure level.

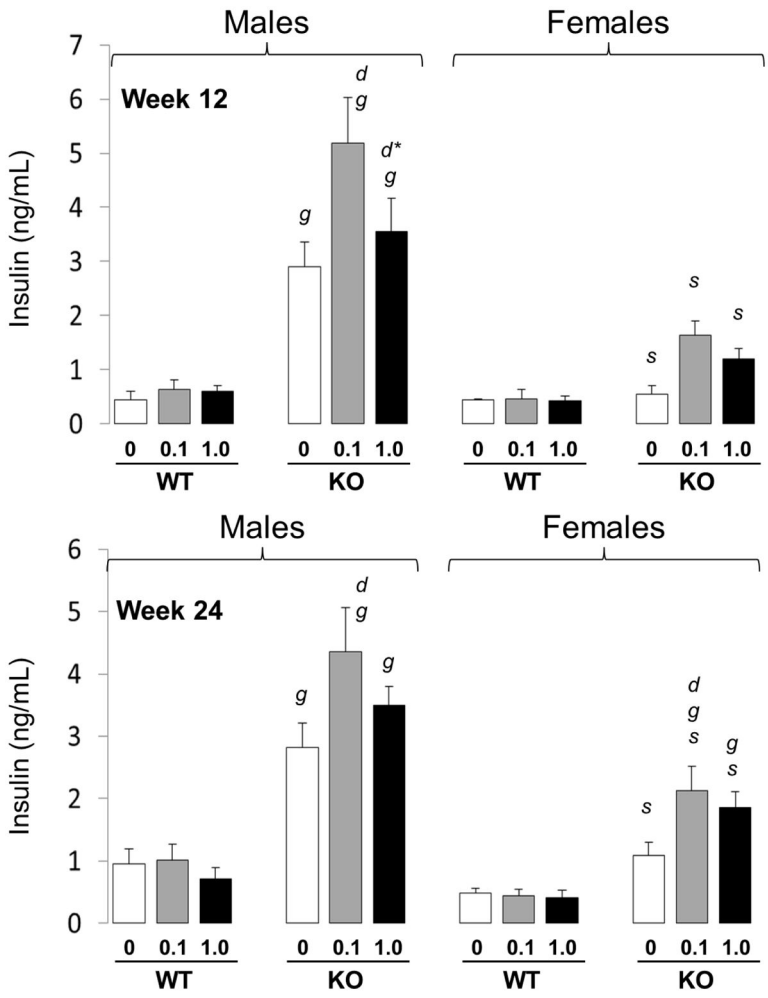


**Figure 3.**

Fasting plasma insulin in male and female wild-type (WT) and *As3mt*-knockout (KO) mice after 12 and 24 weeks of exposure to 0, 0.1, or 1 ppm As in drinking water (Mean +SE, N = 10–14). P<0.05 for the following comparisons: *d*, 0.1 vs 0 ppm or 1 vs 0 ppm for mice of the same sex and genotype; *d**, 1 vs. 0.1 ppm for mice of the same sex and genotype; *g*, KO vs WT mice of the same sex and at the same exposure level; *s*, females vs males of the same genotype and at the same exposure level.

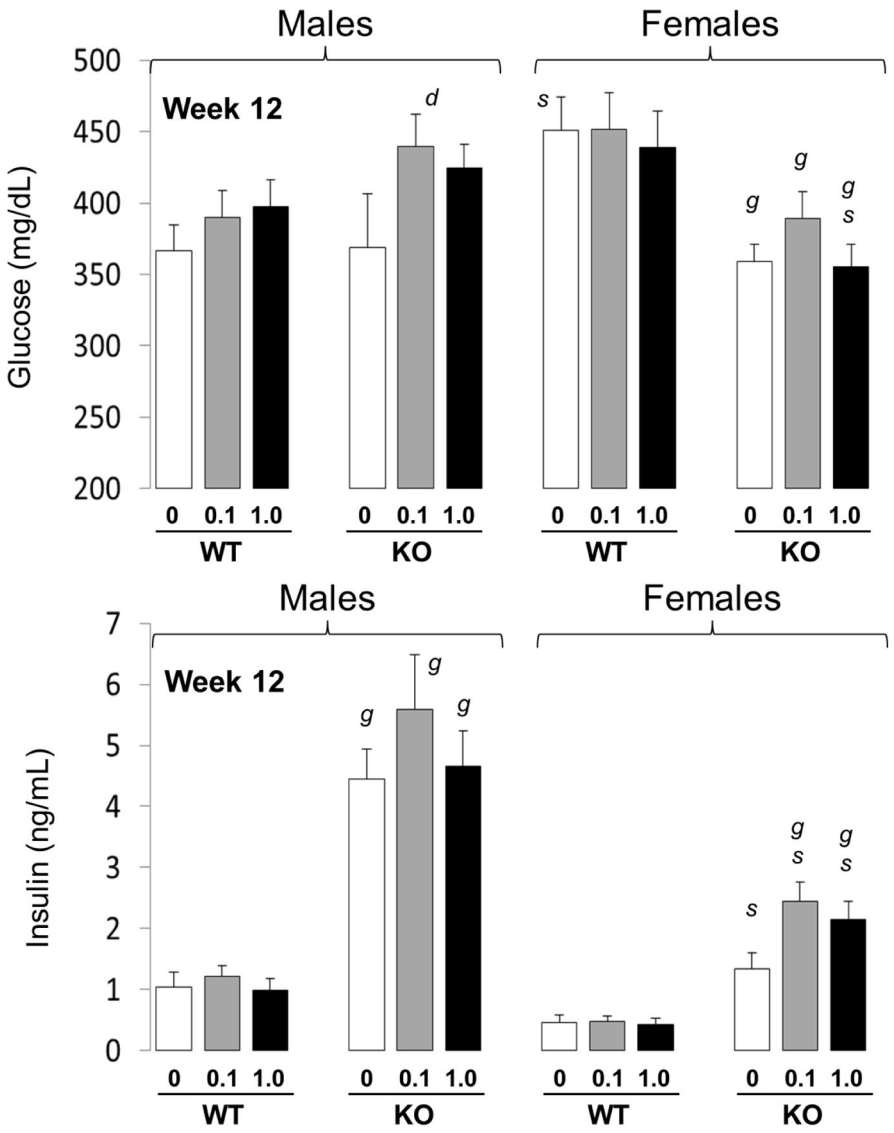


**Figure 4.**

Blood glucose (upper panel) and plasma insulin (lower panel) 15 minutes after i.p. injection of glucose; male and female wild-type (WT) and *As3mt*-knockout (KO) mice were exposed to 0, 0.1, or 1 ppm As in drinking water for 12 weeks (Mean +SE, N = 10–14). P<0.05 for the following comparisons: *d*, 0.1 vs 0 ppm or 1 vs 0 ppm for mice of the same sex and genotype; *g*, KO vs WT mice of the same sex and at the same exposure level; *s*, females vs males of the same genotype and at the same exposure level.

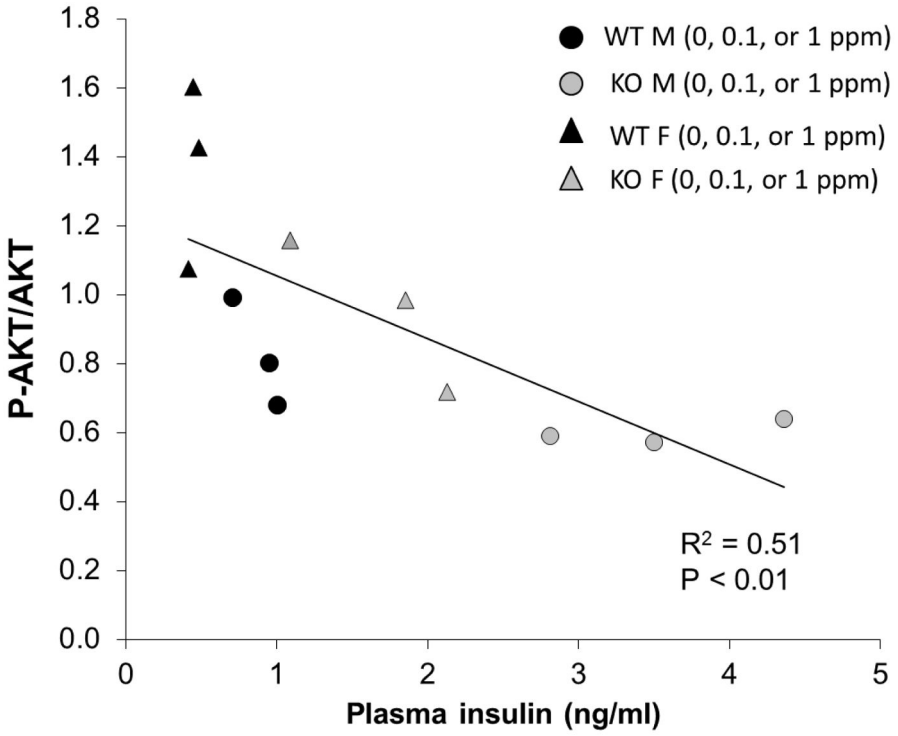


**Figure 5.**

Linear correlation between the average p-Akt/Akt ratio in the liver and the average concentration of insulin in fasting plasma of male (M) and female (F) wild-type (WT) and *As3mt*-knockout (KO) mice after 24-week exposure to 0, 0.1, or 1 ppm As in drinking water.

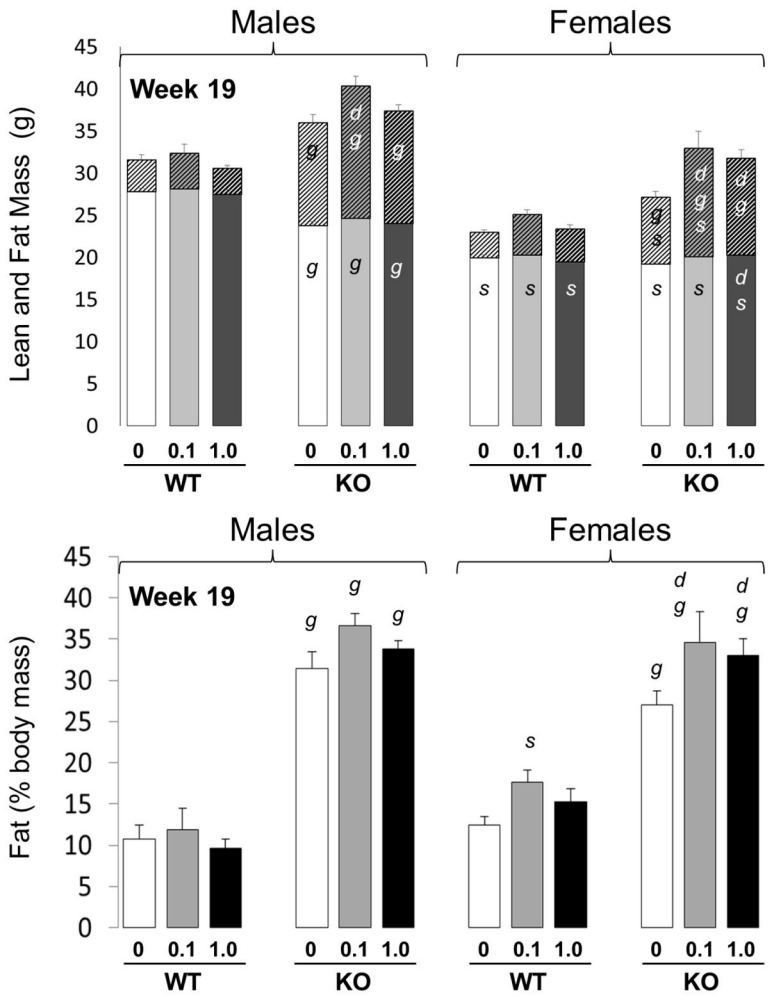


**Figure 6.**

Body composition of male and female wild-type (WT) and *As3mt*-knockout (KO) mice exposed to 0, 0.1, or 1 ppm As in drinking water for 19 weeks (Mean +SE, N = 10–14). Upper panel: lean mass (lower bar) and fat mass (upper hatched bar). Lower panel: % body fat. P<0.05 for the following comparisons: *d*, 0.1 vs 0 ppm or 1 vs 0 ppm for mice of the same sex and genotype; *g*, KO vs WT mice of the same sex and at the same exposure level; *s*, females vs males of the same genotype and at the same exposure level

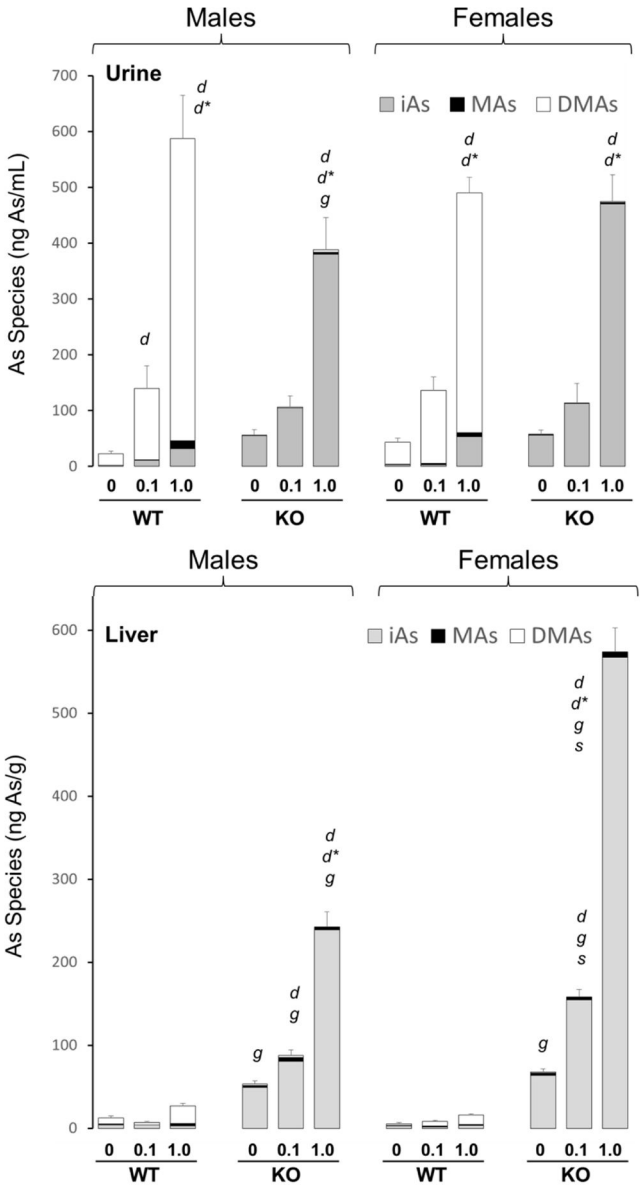


**Figure 7.**

Arsenic species in urine and livers of male and female wild-type (WT) and *As3mt*-knockout (KO) mice after 24 weeks of exposure to 0, 0.1, or 1 ppm As in drinking water (Mean +SE, N = 10–14). P<0.05 for the following comparisons: *d*, 0.1 vs 0 ppm or 1 vs 0 ppm for mice of the same sex and genotype; *d**, 1 vs. 0.1 ppm for mice of the same sex and genotype; *g*, KO vs WT mice of the same sex and at the same exposure level; *s*, females vs males of the same genotype and at the same exposure level.