

# Altered Arsenic Disposition in Experimental Nonalcoholic Fatty Liver Disease

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## ABSTRACT:

Nonalcoholic fatty liver disease (NAFLD) is represented by a spectrum of liver pathologies ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). Liver damage sustained in the progressive stages of NAFLD may alter the ability of the liver to properly metabolize and eliminate xenobiotics. The purpose of the current study was to determine whether NAFLD alters the disposition of the environmental toxicant arsenic. C57BL/6 mice were fed either a high-fat or a methionine-choline-deficient diet to model simple steatosis and NASH, respectively. At the conclusion of the dietary regimen, all mice were given a single oral dose of either sodium arsenate or arsenic trioxide. Mice with NASH excreted significantly higher levels of total arsenic in urine (24 h) compared with controls. Total arsenic in the liver and kidneys of NASH mice was not altered; however, NASH liver retained signifi-

cantly higher levels of the monomethyl arsenic metabolite, whereas dimethyl arsenic was retained significantly less in the kidneys of NASH mice. NASH mice had significantly higher levels of the more toxic trivalent form in their urine, whereas the pentavalent form was preferentially retained in the liver of NASH mice. Moreover, hepatic protein expression of the arsenic biotransformation enzyme arsenic (3+ oxidation state) methyltransferase was not altered in NASH animals, whereas protein expression of the membrane transporter multidrug resistance-associated protein 1 was increased, implicating cellular transport rather than biotransformation as a possible mechanism. These results suggest that NASH alters the disposition of arsenical species, which may have significant implications on the overall toxicity associated with arsenic in NASH.

## Introduction

Nonalcoholic fatty liver disease (NAFLD) is now recognized as the most common cause of liver disease (Marra et al., 2008). It comprises a spectrum of liver pathologies that vary in severity from simple fatty liver (hepatic steatosis) to the more advanced form of nonalcoholic steatohepatitis (NASH), which is characterized by an increase in hepatocellular damage, inflammation, and varying degrees of fibrosis (Ali et al., 2009; Feldstein, 2010). Epidemiological data estimate that NAFLD affects approximately 30 to 40% of the adult population, whereas the prevalence of the more serious form of NASH is esti-

mated to be 5.7 to 17% (McCullough, 2006; Ali and Cusi, 2009). Of particular concern is the fact that up to 25% of NASH patients are reported to develop cirrhosis, and nearly 30 to 40% of these patients perish from a liver-related death over a 10-year period (McCullough, 2006). Given the close association of NAFLD with metabolic disorders such as obesity, dyslipidemia, and insulin resistance, the prevalence of NAFLD is expected to rise near epidemic proportions concomitant with the increasing trend in the worldwide incidence of obesity and type 2 diabetes (Ali and Cusi, 2009).

The exact mechanisms responsible for the pathological progression from hepatic steatosis to NASH are not entirely understood. Factors such as mitochondrial dysfunction, oxidative stress, and proinflammatory cytokine production are central to NASH pathophysiology and are important mediators of the hepatocellular damage and inflammation that is characteristic of the disease (Marra et al., 2008). As a result of the ensuing liver damage seen in NASH, global gene expression is altered, leading to significant dysregulation of liver function (Lake et al., 2011).

The liver plays a crucial role in the metabolism and elimination of many environmental toxicants and clinically relevant drugs from the body. Hepatic-metabolizing enzymes as well as membrane transporters are pivotal in governing the pharmacokinetic fate of many xeno-

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**ABBREVIATIONS:** NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; MMA, monomethyl-arsenic; DMA, dimethyl arsenic; As3mt, arsenic (3+ oxidation state) methyltransferase; HPLC, high-performance liquid chromatography; MCD, methionine and choline deficient; Mrp, multidrug resistance-associated protein; Erk, extracellular signal-regulated kinase; iAs, inorganic arsenic; SAM, (S)-adenosylmethionine.

biotics. The liver damage manifested throughout the progressive stages of NAFLD has been shown to disrupt the expression of clinically important membrane transporters and phase I/III drug-metabolizing enzymes in the liver of both rodent models and human NAFLD (Fisher et al., 2009b; Hardwick et al., 2010, 2011). These alterations in expression have been shown to cause functional aberrations in the hepatobiliary excretion of bromosulphophthalein, acetaminophen metabolites, as well as ezetimibe in a rodent model of NAFLD (Lickteig et al., 2007; Fisher et al., 2009a; Hardwick et al., 2012). These pharmacokinetic disturbances observed in NAFLD can greatly affect the therapeutic efficacy and/or toxicity associated with drugs. Although knowledge is emerging on the functional capacity of the liver to properly eliminate xenobiotics in the context of NAFLD, little information is currently available regarding the effect of NAFLD on the metabolism and elimination of environmental toxicants, such as arsenic.

Arsenic is a toxic metalloid found naturally at various levels in water, soil, and food. Chronic exposure to arsenic, namely via contaminated drinking water, is associated with a host of health-related effects including peripheral vascular diseases and various forms of cancer (Yoshida et al., 2004). Paradoxically, arsenic trioxide has been used for centuries in the management of various diseases including psoriasis and syphilis and is currently approved by the U.S. Food and Drug Administration for the treatment of relapsing acute promyelocytic leukemia (Dilda and Hogg, 2007).

In mammals, inorganic arsenic (iAs) is primarily metabolized in the liver via an oxidative biomethylation scheme sequentially yielding both monomethyl-arsenic (MMA) and dimethyl-arsenic (DMA) metabolites (Drobná et al., 2010). The methylation of arsenic is catalyzed by a single enzyme, arsenic (3+ oxidation state) methyltransferase (As3mt) (Lin et al., 2002), leading to the formation of both trivalent and pentavalent MMA and DMA metabolites, which are predominantly excreted in the urine (Thomas, 2007). Although biomethylation of arsenic was once viewed as a detoxification process, the formation of the intermediate trivalent species is now considered to be an activation mechanism because of the more potent toxicity of these species compared with the pentavalent form (Wang et al., 2007).

There is a limited amount of data concerning how pathophysiological factors such as disease may influence the metabolism and/or excretion of environmental toxicants. The purpose of the current study was to determine whether experimentally induced NAFLD in mice alters the disposition of arsenic. Sodium arsenate was used to model environmental exposure, whereas arsenic trioxide was used to replicate clinical exposure in this study. Understanding the effects of liver disease on arsenic disposition may have important implications in predicting toxicity and/or efficacy in individuals with NASH.

### Materials and Methods

**Materials.** Sodium arsenate (acid heptahydrate sodium salt) and high-performance liquid chromatography (HPLC)-grade H<sub>2</sub>O were obtained from Thermo Fisher Scientific (Waltham, MA). Arsenic trioxide was a kind gift from Michael J. Kopplin at the University of Arizona (Tucson, AZ). Both arsenical compounds used in dosing were determined to be >99.8% pure. HClO<sub>4</sub>, HgCl<sub>2</sub>, KOH, Tris-HCl, EDTA, NaCl, glycerol, and octyl phenoxypolyethoxyethanol (Nonidet P-40) were obtained from Sigma-Aldrich (St. Louis, MO).

**Animals.** Male C57BL/6 mice weighing 20 to 25 g were obtained from Harlan (Indianapolis, IN). All animals were acclimated in 12-h light and dark cycles in a University of Arizona Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facility for 1 week before initiation of experiments and were allowed standard chow and water ad libitum. Housing and experimental procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory*

*Animals* (Institute of Laboratory Animal Resources, 1996). Mice were fed an isocaloric diet ( $n = 3$ ) (number 180820) as a control or a high-fat diet ( $n = 5$ ) (high cholesterol, 18% butter fat) diet (number 112280) for 5 weeks to induce simple steatosis (Dyets, Inc., Bethlehem, PA). Mice were placed on a methionine- and choline-deficient (MCD) diet ( $n = 5$ ) (number 518810) for 8 weeks to induce NASH or a methionine and choline resupplemented diet ( $n = 3$ ) (number 518754) as a control (Dyets, Inc., Bethlehem, PA). Because no histopathological changes were observed between both control diets (isocaloric and methionine-choline resupplemented diets), they were both combined as a single control group in statistical analyses for all experiments conducted in this study.

**Arsenic Disposition Study.** After the time allotted on the respective diets, the mice were given a single oral dose of either 0.75 mg/kg sodium arsenate or 0.2 mg/kg arsenic trioxide dissolved in HPLC-grade H<sub>2</sub>O. Dosing was performed using 20-gauge feeding needles (Thermo Fisher Scientific). After dosing, the mice were placed in Nalgene metabolic cages (PGC International, Palm Desert, CA), where total urine was collected for 24 h. At the conclusion of the 24-h period, the mice were euthanized by CO<sub>2</sub> asphyxiation, followed by collection of livers and kidneys. A section of each liver was cut and fixed with 10% neutral-buffered formalin overnight, followed by paraffin embedding for hematoxylin and eosin staining. The remaining liver, along with kidneys, was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. Total urine was transferred to a sterile collection tube and stored at  $-80^{\circ}\text{C}$  before analysis.

**Sample Preparation.** The extraction of arsenical species from liver and kidney was performed using previously described methods (Csanaky and Gregus, 2003). All reagents were prepared in HPLC-grade H<sub>2</sub>O. Approximately 200 mg of tissue was homogenized in 1 ml of ice-cold HClO<sub>4</sub> (0.4 M). Five hundred microliters of homogenate was transferred to a sterile collection tube containing 50  $\mu\text{l}$  of aqueous 150 mM HgCl<sub>2</sub> and then gently mixed and kept on ice for 1 min. The samples were centrifuged at 15,850g for 2 min at 4°C. The supernatant was removed and transferred to a sterile collection tube, and the pH was adjusted to  $\sim 2$  using 1 M KOH. Samples were centrifuged at 15,850g for 2 min at 4°C, and the supernatant was removed and stored at  $-80^{\circ}\text{C}$  before analysis for arsenic speciation. It must be noted that although these samples were treated with HClO<sub>4</sub> and HgCl<sub>2</sub> per the extraction method, there is a lack of evidence regarding the effect of these compounds on the arsenic oxidation state present in the tissue homogenates. Therefore, there may be an effect of these treatments on the valence state of arsenic, and it may not accurately reflect the levels of trivalent and pentavalent arsenic present in the unperturbed tissue. However, because all the samples were treated and processed in the same manner, it is conceivable that any difference in the pattern of arsenic oxidation state across the samples tested can be explained not by HgCl<sub>2</sub> and HClO<sub>4</sub> treatment but by the inherent differences in the tissue samples themselves.

**Determination of Arsenic Species in Urine and Tissue.** The arsenic speciation method is adapted from Gong et al. (2001). Urine and previously extracted tissue homogenates were filtered through 0.45- $\mu\text{m}$  nylon centrifuge filters and were diluted before injection into the HPLC system. The HPLC system consisted of an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA) with a reverse-phase C18 column (Gemini 5  $\mu\text{m}$  C18 110 Å, 150  $\times$  4.60 mm; Phenomenex, Torrance, CA) and guard cartridge. The mobile phase (pH 5.85) contained 4.7 mM tetrabutylammonium hydroxide, 2 mM malonic acid, and 4% (v/v) methanol at a flow rate of 1.2 ml/min. The column temperature was maintained at 50°C, and samples were kept at 4°C in a thermally controlled autosampler. An Agilent 7500ce inductively coupled plasma mass spectrometer (series ICP-MS; Agilent Technologies) with a Conikal nebulizer (Glass Expansion, Pocasset, MA) was used as the detector. The operating parameters were as follows: radiofrequency power, 1500 W; plasma gas flow, 15 l/min; and carrier flow,  $\sim 0.9$  l/min, 0.15 l/min makeup; and arsenic was measured at 75 m/z.

**Protein Preparations.** Whole-cell lysate preparations of mouse liver were prepared from  $\sim 250$  mg of tissue homogenized in NP-40 buffer [20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA with one Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN) per 50 ml] at 4°C. Homogenized tissue was then agitated at 4°C for 2 h and centrifuged at 10,000g for 30 min, and the supernatant was transferred to a clean collection tube. Protein concentrations were determined

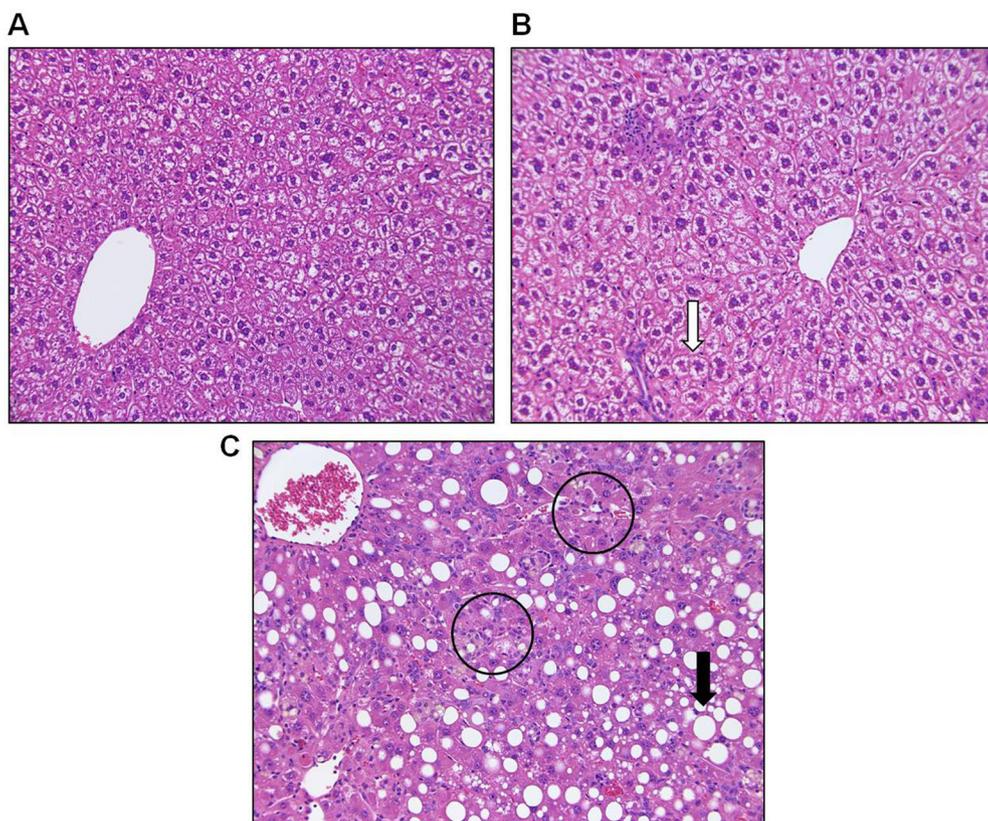


FIG. 1. Liver histopathology of mice fed a control, high-fat and MCD diet. Representative hematoxylin and eosin-stained liver sections from control mice (A), mice fed high-fat diet (B), and mice fed MCD diet (C). Microvesicular steatosis identified in the mice fed a high-fat diet is indicated by the white arrow, whereas macrovesicular steatosis after a MCD diet is shown by the black arrow. Inflammation is clearly visible in MCD-fed mice, evident by the presence of inflammatory cells (circle). Images were taken at  $20\times$  magnification.

using the Pierce BCA Protein Quantitation Assay (Thermo Fisher Scientific) per the manufacturer's protocol.

**Immunoblot Protein Analysis.** Whole-cell lysate proteins ( $75\ \mu\text{g}/\text{well}$ ) were prepared in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with  $\beta$ -mercaptoethanol and were heated at  $90^\circ\text{C}$  for  $\sim 7$  min before separation by SDS-polyacrylamide gel electrophoresis on 10% gels. Proteins were transferred to polyvinylidene fluoride membranes overnight at  $4^\circ\text{C}$ . After transfer, the membranes were blocked in 5% nonfat dry milk diluted in either phosphate-buffered saline/Tween 20 [multidrug resistance-associated protein 1 (Mrp1)] or Tris-buffered saline/Tween 20 (As3mt) for 45 min at room temperature. To determine relative protein levels of As3mt, a rabbit polyclonal antibody, developed with full-length human As3MT and containing cross-reactivity toward both rat and mouse As3mt, was used (kindly donated by Walter Klimecki at the University of Arizona, Tucson, AZ). Relative protein levels of Mrp1 were performed using a rat monoclonal antibody against Mrp1 (clone MRPr1; generated by George L. Scheffer, Amsterdam, The Netherlands) with known cross-reactivity against both human and mouse Mrp1 (Aleksunes et al., 2008). The blots were incubated with primary antibody overnight at  $4^\circ\text{C}$  with constant rocking. The following horseradish peroxidase-conjugated secondary antibodies were used: anti-rat (sc-2065) and anti-rabbit (sc-2004) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Quantification of relative protein expression was determined using image processing and analysis with ImageJ software (National Institutes of Health, Bethesda, MD) and was normalized to extracellular signal-regulated kinase (Erk) 1 protein (C-16; Santa Cruz Biotechnology, Inc.). Erk protein has been previously validated and used to normalize protein expression data from NASH samples (Hardwick et al., 2010). The use of more customary proteins such as glyceraldehyde-3-phosphate dehydrogenase has been shown to be inconsistent, perhaps because of the association of NASH with fibrosis.

**Statistical Analysis.** Data were analyzed using one-way analysis of variance to determine significant findings among diet groups with a Bonferroni post hoc analysis. A two-way analysis of variance was used to determine significance between arsenic metabolites found in urine and tissue among the diet groups followed by a Bonferroni post hoc analysis. A significance level of  $p \leq 0.05$  was used for all analyses. All analyses were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

## Results

**Mouse Liver NAFLD Histology.** Representative images of hematoxylin and eosin-stained liver sections from mice fed a control diet, high-fat diet, and MCD diet are shown in Fig. 1. Microvesicular lipid deposits are clearly visible in the liver of mice fed a high-fat diet for 5 weeks (white arrowhead), whereas macrovesicular steatosis was primarily observed within the livers of MCD-fed mice (black arrowheads). Inflammation, a key distinguishing feature of NASH, is present in the livers of MCD-fed mice (circles). These lesions are consistent with the hepatic histopathological progression of human NAFLD from simple fatty liver (steatosis) to the more advanced form of NASH, and the histopathological assessment of these two dietary models in rodents has been characterized previously by a veterinary pathologist at the University of Arizona using a validated NAFLD scoring system (Kleiner et al., 2005).

**Urinary Excretion of Arsenic in Experimental NAFLD.** Cumulative 24-h arsenic excretion in the urine of control, steatosis, and NASH mice is shown in Fig. 2. Mice with NASH excreted a significantly higher proportion ( $p < 0.05$ ) of total arsenic in 24 h compared with control after a single oral dose of  $0.2\ \text{mg}/\text{kg}$  arsenic trioxide (Fig. 2A). No statistical difference in the excretion of total arsenic was observed between steatosis mice and controls. Mice with NASH given a single oral dose of  $0.75\ \text{mg}/\text{kg}$  sodium arsenate did not excrete significantly higher levels of arsenic in their urine compared with control (Fig. 2B).

A breakdown of the relative amount of arsenic metabolites excreted in 24-h urine of control, steatosis, and NASH mice dosed with arsenic trioxide and sodium arsenate is shown in Fig. 3. The DMA metabolite was the predominant metabolite recovered in the urine of both treatment groups, and its excretion was not affected by disease state. However, mice with NASH excreted significantly less ( $p < 0.05$ ) iAs compared with controls after arsenic trioxide treatment (Fig. 3A). No difference in the

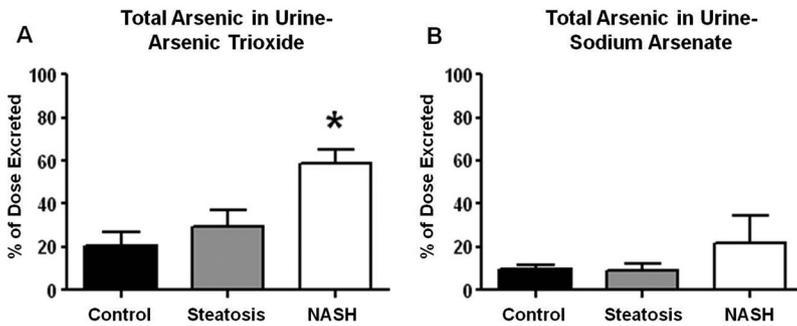


FIG. 2. Total arsenic elimination in urine. Percentage of total arsenic dose excreted in 24-h urine from control, steatosis, and NASH mice given a single oral dose of arsenic trioxide (A) or sodium arsenate (B). Data represent the mean  $\pm$  S.D. from four to six mice. \*,  $p < 0.05$  versus control mice.

relative amounts of metabolites excreted in 24-h urine was detected among disease groups in animals treated with sodium arsenate (Fig. 3B).

To determine whether the valence state had an effect on the disposition of arsenic metabolites in NASH, the amount of trivalent and pentavalent iAs was quantified. Mice with NASH were found to differentially excrete trivalent and pentavalent iAs in their urine (Fig. 4). Specifically, mice with NASH excreted a statistically higher ( $p < 0.05$ ) percentage of the dose as trivalent iAs compared with control after a single oral dose of arsenic trioxide (Fig. 4B). However, urinary excretion of trivalent iAs in NASH versus control mice given sodium arsenate did not reach statistical significance (Fig. 4D). No change in the excretion of pentavalent iAs in urine was observed among disease states (Fig. 4, A and C).

**Renal and Hepatic Accumulation of Arsenic in Experimental NAFLD.** Arsenic accumulation in the liver and kidneys of mice was evaluated 24 h after dosing with either arsenic trioxide or sodium arsenate to determine disease-dependent retention of arsenical species in these tissues. No difference in total arsenic accumulation in the liver was identified among disease groups (Fig. 5, A and C). In contrast, the accumulation of specific arsenic metabolites was altered in a disease-dependent fashion (Fig. 5, B and D). Mice with NASH that were dosed with arsenic trioxide and sodium arsenate accumulated significantly higher ( $p < 0.05$ ) MMA levels in liver compared with controls and showed a significant reduction in the level of DMA.

Accumulation of total arsenic and arsenic metabolites in the kidneys of control, steatosis, and NASH mice is shown in Fig. 6. No significant difference in the renal levels of total arsenic was identified in the kidneys 24 h after dosing for either arsenic trioxide or sodium arsenate between disease groups (Fig. 6, A and C). However, similar to what was observed in the liver, the retention of arsenic metabolites in kidney was altered in a disease-dependent fashion (Fig. 6, B and D). Specifically, retention of iAs was significantly increased ( $p < 0.05$ ) with a concomitant decrease ( $p < 0.05$ ) in the renal levels of DMA in mice with NASH treated with arsenic trioxide and sodium arsenate.

To further investigate the effects of NAFLD on the disposition of arsenic, the retention of both trivalent and pentavalent iAs was mea-

sured in livers of control, steatosis, and NASH mice 24 h after dosing with either arsenic trioxide or sodium arsenate (Fig. 7). NASH livers had a significantly higher proportion ( $p < 0.05$ ) of the iAs content as pentavalent iAs compared with control (Fig. 7, A and C). Conversely, no statistically significant difference in the levels of trivalent iAs was observed among disease groups in both arsenic trioxide- and sodium arsenate-treated mice (Fig. 7, B and D).

**As3mt and Mrp1 Expression in Mouse Liver.** To explore a possible mechanism responsible for the altered disposition of arsenic metabolites, metabolizing enzyme and transporter protein levels were measured in the livers of control, steatosis, and NASH mice (Fig. 8A). No significant change in relative protein expression of As3mt was found between control and NASH mice; however, As3mt protein was significantly ( $p < 0.05$ ) down-regulated in the livers of mice with steatosis compared with control. Furthermore, the protein expression of Mrp1, a membrane transporter known to transport arsenical species, was significantly ( $p < 0.05$ ) induced in the livers of mice with steatosis and NASH compared with control.

## Discussion

Millions of people worldwide are exposed to high levels of arsenic, placing them at risk for developing various forms of cancer as well as nonmalignant diseases (Thomas et al., 2001; Rosen and Liu, 2009). With the extensive worldwide prevalence of NAFLD, it is reasonable to suspect that individuals exposed to high levels of arsenic may also have NAFLD. However, little information is known regarding the effect of NAFLD on the fate of arsenic within the body.

The purpose of the current study was to determine whether experimentally induced NAFLD alters the normal metabolism, disposition, and/or excretion of the environmental toxicant arsenic. Our results clearly demonstrate that mice with the most progressive form of NAFLD, NASH, were found to excrete significantly more total arsenic in their urine after a single oral dose of arsenic trioxide. A similar profile was seen in mice administered sodium arsenate; however, the results did not reach statistical significance. Previous studies investi-

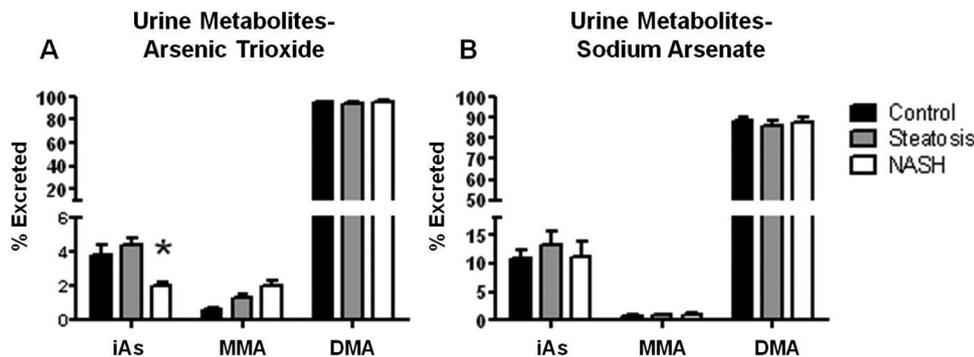


FIG. 3. Urine profile of arsenic metabolites. Relative amount of arsenic metabolites recovered in 24-h urine from control, steatosis, and NASH mice given a single oral dose of arsenic trioxide (A) or sodium arsenate (B). Data represent the mean  $\pm$  S.D. from four to six mice. \*,  $p < 0.05$  versus control mice.

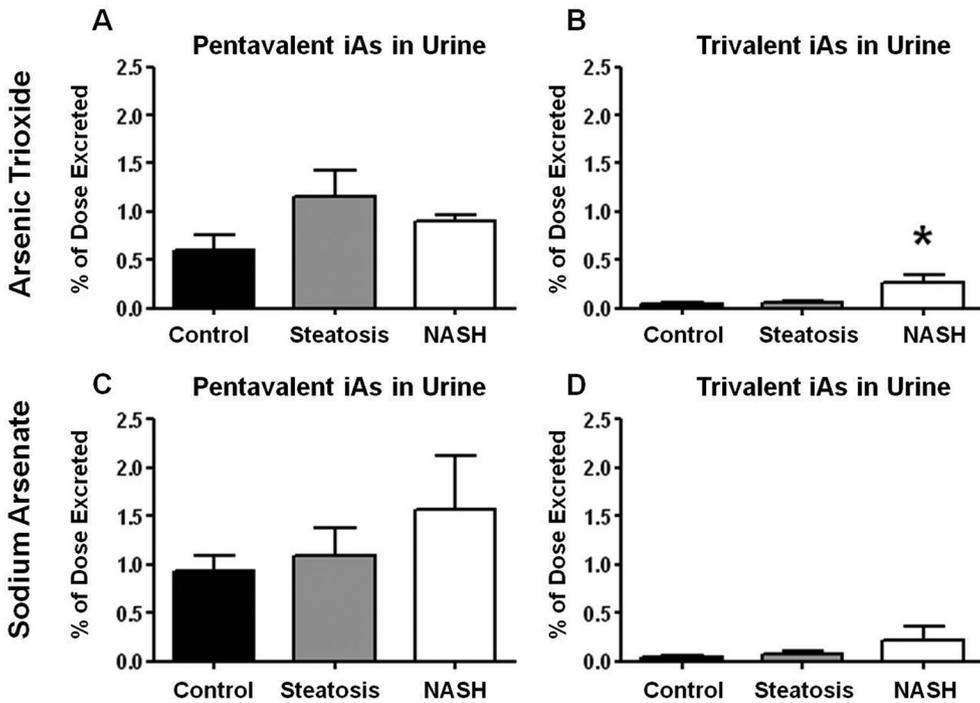


FIG. 4. Differential elimination of trivalent and pentavalent iAs in urine. Percentage of the arsenic dose excreted as pentavalent (A and C) and trivalent (B and D) iAs in 24-h urine from control, steatosis, and NASH mice. Mice were given either a single oral dose of arsenic trioxide (A and B) or sodium arsenate (C and D). Data represent the mean  $\pm$  S.D. from four to six mice. \*,  $p < 0.05$  versus control mice.

gating the effects of hepatitis, steatosis, and alcoholic cirrhosis in humans have also reported an increase in the urinary elimination of arsenic compared with healthy individuals (Buchet et al., 1984). This suggests that the manifestation of disease in the liver may have a considerable influence on the elimination of this toxic metalloid.

In addition to the intrinsic liver damage sustained throughout the progressive stages of NAFLD, associated metabolic disorders such as obesity may further affect the elimination of arsenic. A study conducted in a female population in southwest Arizona and northern Mexico determined a positive correlation between arsenic methylation efficiency and body mass index (Gomez-Rubio et al., 2011). Specifically, a lower MMA coupled to a higher DMA/MMA ratio was measured in the urine of these women. In contrast, our results suggest

that although total arsenic elimination is increased in mice with NASH, no significant aberration in methylation efficiency was observed. These findings may be partly explained by the observation that the majority of metabolites recovered in the urine of mice were dimethylated, suggesting higher methylation efficiencies in these animals.

Arsenic is primarily metabolized in the liver by As<sub>3</sub>mt and uses the methyl donor (*S*-adenosylmethionine (SAM) to produce both mono and dimethyl arsenic metabolites (Lin et al., 2002). To investigate a potential mechanism for the observed increase in arsenic found in the urine of mice with NASH, we measured relative protein expression of As<sub>3</sub>mt in the liver. No significant change in protein expression of hepatic As<sub>3</sub>mt was detected in NASH animals, suggesting that the

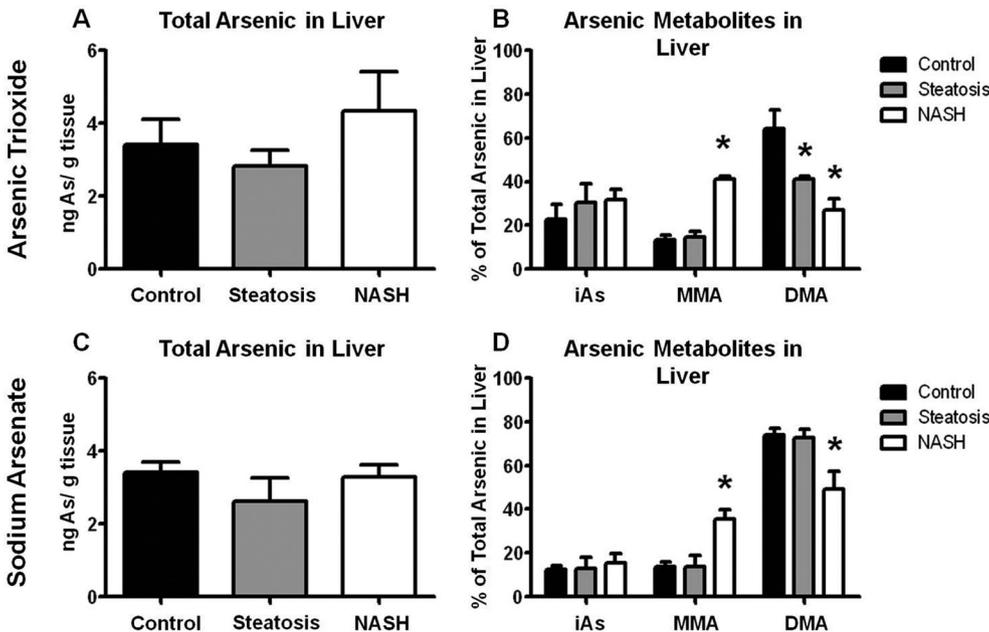


FIG. 5. Liver accumulation of arsenic metabolites. Total arsenic retained in the livers of control, steatosis, and NASH mice 24 h after being given a single oral dose of arsenic trioxide (A and B) or sodium arsenate (C and D). A and C depict total arsenic retained in liver, whereas B and D depict the percentage of arsenic species present in the liver 24 h after the dose. Data represent the mean  $\pm$  S.D. from four to six mice. \*,  $p < 0.05$  versus control mice.

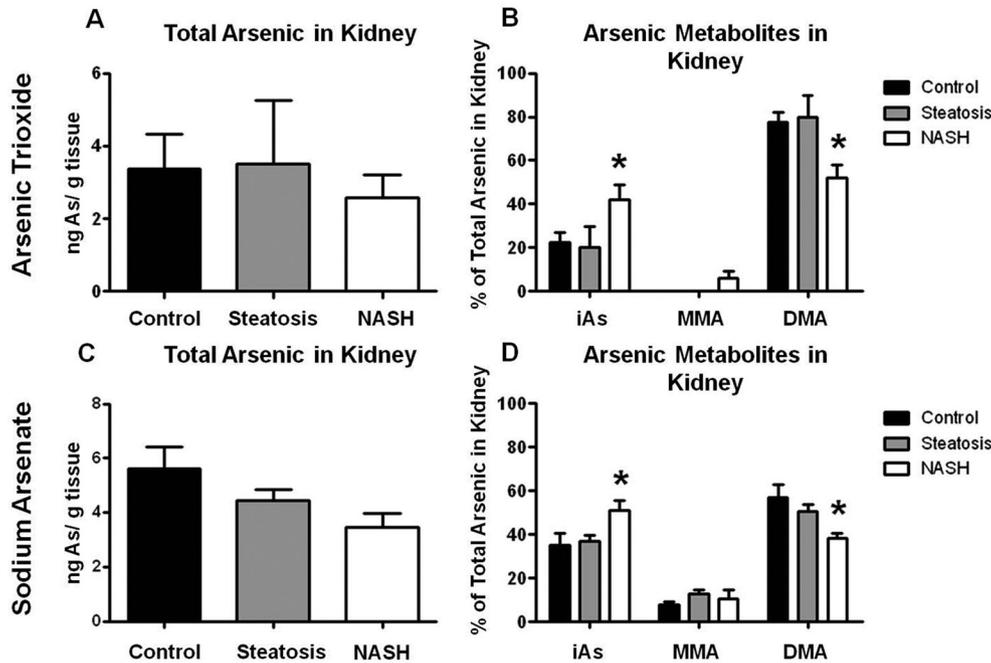


FIG. 6. Kidney accumulation of arsenic metabolites. Total arsenic retained in the kidneys of control, steatosis, and NASH mice 24 h after being given a single oral dose of arsenic trioxide (A and B) or sodium arsenate (C and D). A and C depict total arsenic retained in kidneys. B and D depict percentage of arsenic species present in the kidneys 24 h after the dose. Data represent the mean  $\pm$  S.D. from four to six mice. \*,  $p < 0.05$  versus control mice.

increase in total urinary excretion is not due to alterations in metabolism. These findings coincide with the observation that the relative amounts of both monoethylated and dimethylated arsenic metabolites recovered in the urine were not altered by disease state. A significant down-regulation of As3mt protein expression was seen in mice with steatosis without any concomitant aberration in total arsenic, MMA, or DMA recovery in the urine. However, a decrease in DMA was present in livers of mice with steatosis. A potential explanation for the observation that no differential effects on arsenic elimination were seen in steatosis mice, despite the down-regulation in As3mt expression, may lie in alternative methods for arsenic methylation *in vivo*. A study using As3mt-knockout mice given an oral dose of arsenate revealed the presence of fully dimethylated metabolites in urine (Drobna et al., 2009). These results suggest that alternative mechanisms for arsenic methylation may exist, which may partially com-

pensate for the decrease in As3mt expression seen in mice with steatosis. In addition, presystemic metabolism by gut microflora may further contribute to the appearance of methylated urinary metabolites in the current study. Various microorganisms normally found in the gastrointestinal tract have the capacity to methylate arsenic (Bentley and Chasteen, 2002). Nonetheless, these data suggest for the first time that As3mt protein expression may be altered in different pathophysiological states. Further investigation is needed to identify whether dysregulation of As3mt protein expression also occurs in humans with NASH.

In addition to the direct effects of protein expression on As3mt function, aberrations in the levels of the necessary methyl donor SAM may indirectly alter the function of this enzyme. The amino acid methionine is a necessary cofactor in the biosynthesis of SAM (Loenen, 2006), and dietary intake of methionine has been shown to

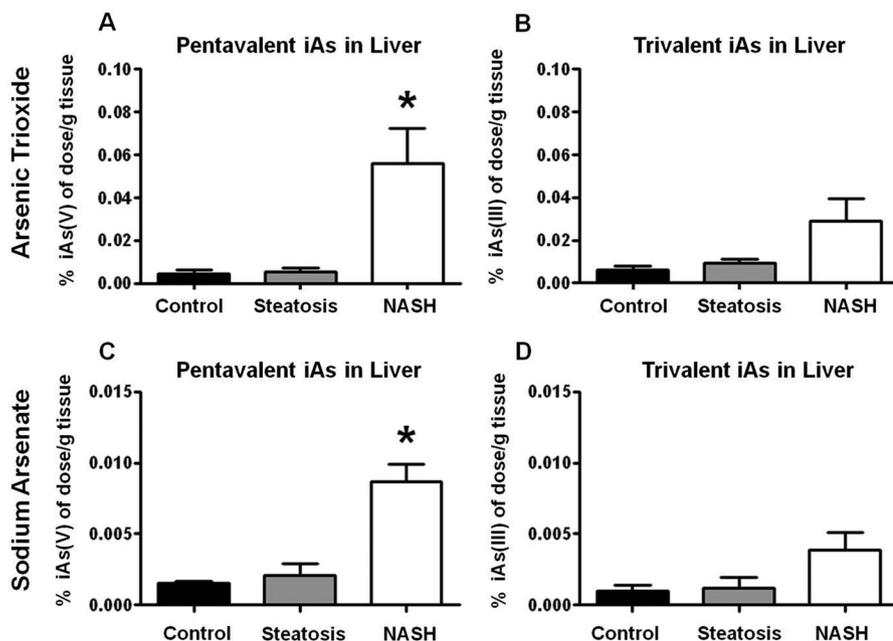


FIG. 7. Differential accumulation of arsenic metabolites in liver. Percentage of the arsenic dose retained in the liver as pentavalent (A and C) and trivalent (B and D) iAs 24 h after the arsenic dose from control, steatosis, and NASH mice. Mice were given a single oral dose of arsenic trioxide (A and B) or sodium arsenate (C and D). Data represent the mean  $\pm$  S.D. from four to six mice. \*,  $p < 0.05$  versus control mice.

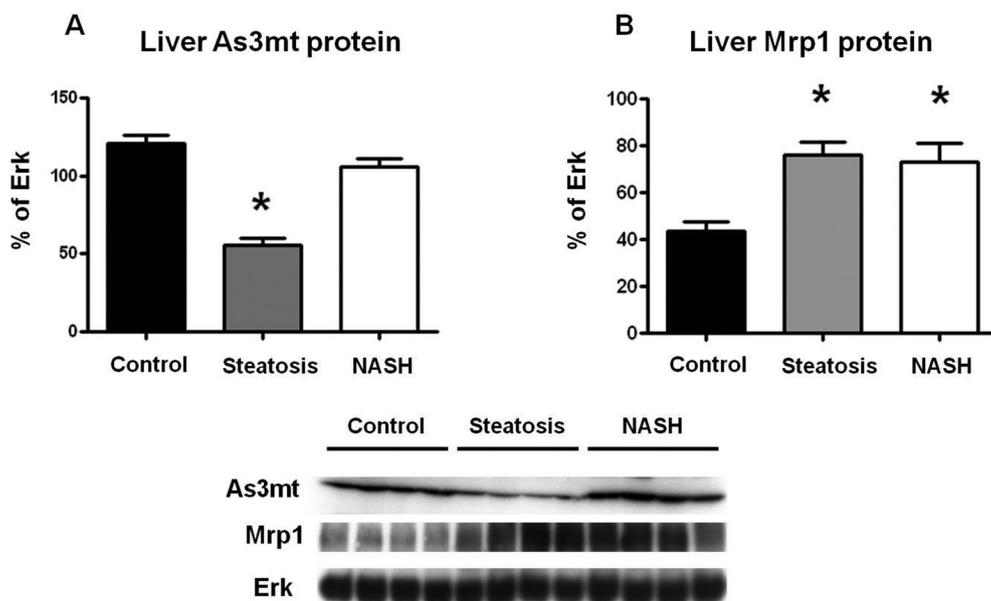


FIG. 8. Hepatic protein expression of As3mt and Mrp1. Relative protein expression of As3mt (A) and Mrp1 (B) in the liver of control, steatosis, and NASH mice. Data are normalized to Erk protein expression. Data represent the mean  $\pm$  S.D. from four to six mice. \*,  $p < 0.05$  versus control mice.

affect the methylation efficiency of arsenic in humans and animals (Heck et al., 2009; Jin et al., 2010). In addition, the effects of the MCD diet on hepatic SAM levels in mice remain controversial because some groups have reported depletion in hepatic SAM, whereas others report no change in hepatic SAM after 6 weeks of MCD diet (Gyamfi et al., 2008; Caballero et al., 2010). However, our results show that despite the depletion in dietary methionine, the majority of excreted arsenic recovered in the urine from mice fed the MCD diet was dimethylated. This observation suggests that methylation efficiency was not impaired by the MCD diet in light of the potential confounding influences of SAM depletion. Prior evidence from rabbits fed diets deficient in methionine or choline showed a decrease in the urinary excretion of methylated arsenic metabolites coupled with higher tissue retention of arsenic in a 72-h period (Vahter and Marafante, 1987). In contrast, the results presented in this study do not suggest that dietary methionine and choline depletion in mice alters the methylation capacity of arsenic because no change in total hepatic arsenic retention or urinary excretion of arsenic metabolites was observed. Lower rates of methionine transmethylation have been shown to occur in humans with NASH, implicating a lower rate of SAM biosynthesis in this population of individuals (Kalhan et al., 2011). Whether this alters the metabolism of arsenic in humans with NASH is unknown.

A key factor in the overall toxicity of arsenic is the formation of the trivalent MMA and DMA metabolites, which have been shown to be significantly more toxic than their pentavalent counterparts (Kligerman et al., 2003; Wang et al., 2007). The identification of these toxic metabolites in human urine suggests that they are sufficiently stable to distribute into target tissues and elicit a toxicologic response (Mandal et al., 2001). Because of limitations and restrictions of the current analysis, we could not determine the valence state of the methylated species in the urine or tissues sampled. However, this analysis was able to differentiate and quantify pentavalent and trivalent iAs. Interestingly, we identified a significant shift in the renal elimination and liver accumulation of these arsenical species between different disease groups. Mice with NASH had significantly higher levels of the more toxic trivalent iAs in urine, whereas livers accumulated significantly higher levels of the pentavalent form.

The role of membrane transporters in regulating the disposition of arsenic is slowly emerging as an important mechanism in governing

its disposition. In particular, one member of the ABCB subfamily of efflux transporters, the Mrp1, has been implicated in the cellular transport of arsenic (Lorico et al., 2002; Thomas, 2007). Mrp1 has been shown to transport arsenite<sup>III</sup> but not arsenate<sup>V</sup> in the presence of glutathione (Leslie et al., 2004), suggesting that transport of arsenic by Mrp1 is dependent on the valence state, with Mrp1 preferentially transporting trivalent iAs over its pentavalent counterpart. In addition, an Mrp1-overexpressing cell line has been shown to confer resistance to trivalent MMA but not pentavalent MMA in a GSH-dependent fashion, suggesting that trivalent MMA is also a Mrp1 substrate and further lending support to the valence specificity of arsenic transporters (Carew et al., 2011). To investigate whether the increase in elimination of trivalent iAs in urine of mice with NASH could be partially explained by dysregulation of Mrp1, we measured its relative protein expression in the liver. Our results indicate that Mrp1 is significantly induced in the liver of mice with NASH and steatosis, suggesting a possible mechanism for the increase in trivalent iAs urinary excretion observed in NASH mice. Furthermore, arsenic is a known substrate for the hepatobiliary transporter Mrp2, and its function is required for the biliary elimination of arsenic-GSH conjugates (Kala et al., 2000, 2004; Carew and Leslie, 2010). Previous reports have demonstrated that Mrp2 localization on the bile canalicular membrane is altered in both human and rodent models of NASH, thus inhibiting its ability to properly excrete compounds into the bile (Hardwick et al., 2011, 2012). However, further investigation is needed to confirm whether biliary excretion of arsenical metabolites is altered in NASH.

Future investigations need to be conducted to determine the functional and toxicological effects of altered arsenic disposition in NAFLD. In this study, we have demonstrated that NASH increases total arsenic elimination in the urine, along with specific alterations identified in the elimination and tissue accumulation of arsenic metabolites. However, whether these aberrations manifest in an altered toxicodynamic and/or pharmacodynamic effect is yet to be determined. We have identified specific molecular alterations that may provide novel insights into the mechanisms by which NAFLD alters the disposition of arsenic while emphasizing the importance of membrane transporters in influencing the pharmacokinetics of this toxic metalloid.

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### Authorship Contributions

*Participated in research design:* Canet, Gandolfi, and Cherrington.  
*Conducted experiments:* Canet, Hardwick, Lake, and Kopplin.  
*Contributed new reagents or analytic tools:* Scheffer and Klimecki.  
*Performed data analysis:* Canet and Cherrington.  
*Wrote or contributed to the writing of the manuscript:* Canet and Cherrington.

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