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Altered Arsenic Disposition in Experimental Nonalcoholic Fatty Liver Disease

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Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; As3mt; arsenic (3+ oxidation state) methyltransferase; iAs, inorganic arsenic; MMA, monomethyl-arsenic; DMA, dimethyl-arsenic; Mrp1, Multidrug-resistance associated protein 1; MCD, methionine and choline deficient; SAM, S-adenosyl methionine.

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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 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 (24h) compared to controls. Total arsenic in the liver and kidneys of NASH mice was not altered; however NASH liver retained significantly higher levels of the monomethyl (MMA) arsenic metabolite, whereas dimethyl arsenic (DMA) was retained significantly less in the kidneys of NASH mice. NASH mice had significantly higher levels of the more toxic trivalent form (iAs^{III}) in their urine, whereas the pentavalent form (iAs^{V}) was preferentially retained in the liver of NASH mice. Moreover, hepatic protein expression of the arsenic biotransformation enzyme As3mt was not altered in NASH animals whereas protein expression of the membrane transporter Mrp1 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.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is now recognized as the most common cause of liver disease (Marra *et al.*, 2008). It is comprised of 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 (Feldstein, 2010;Ali *et al.*, 2009). Current epidemiological data estimates that NAFLD affects approximately 30-40% of the adult population, while the prevalence of the more serious form of NASH is estimated to be 5.7-17% (Ali and Cusi, 2009;McCullough, 2006). Of particular concern is the fact that up to 25% of NASH patients are reported to develop cirrhosis, and nearly 30-40% of these patients perish from a liver-related death over a ten 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 pro-inflammatory 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 xenobiotics. 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/II drug metabolizing enzymes in the liver of both rodent models and human NAFLD (Hardwick *et al.*, 2011;Fisher *et al.*, 2009b;Hardwick *et al.*, 2010). Importantly, these alterations in expression have been shown to cause functional aberrations in the hepatobiliary excretion of bromosulfophthalein, acetaminophen metabolites as well as ezetimibe in a rodent model of NAFLD (Fisher *et al.*, 2009a;Lickteig *et al.*, 2007;Hardwick *et al.*, 2012). These pharmacokinetic disturbances observed in NAFLD can greatly impact 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 US Food and Drug Administration for the treatment of relapsing acute promyelocytic leukemia (Dilda *et al.*, 2007).

In mammals, inorganic arsenic is primarily metabolized in the liver via an oxidative biomethylation scheme sequentially yielding both monomethyl-As (MMA) and dimethyl-As (DMA) metabolites (Drobna *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 detoxication process, the formation of the intermediate trivalent species is now considered to be an activation mechanism due to the more potent toxicity of these species compared to the pentavalent form (Wang *et al.*, 2007).

There is currently 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 HPLC-grade H_2O was obtained from Fisher Scientific (Pittsburgh, PA). Arsenic trioxide was a kind gift from Michael J. Kopplin at the University of Arizona. Both arsenical compounds used in dosing were determined to be >99.8% pure. $HClO_4$, $HgCl_2$, KOH, Tris-HCl, EDTA, NaCl, glycerol, and Nonidet P-40 were obtained from Sigma-Aldrich (St. Louis, MO).

Animals. Male C57BL/6 mice weighing 20-25 grams were obtained from Harlan Laboratories (Indianapolis, IN). All animals were acclimated in 12 hour light and dark cycles in a University of Arizona AAALAC-certified animal facility for one week prior to initiation of experiments and were allowed standard chow and water *ad libitum.* Housing and experimental procedures were in accordance with NIH guidelines for the care and use of experimental animals. Mice were fed an isolcaloric diet (N=3) (#180820) as a control or a high fat diet (N=5) (high cholesterol, 18% butter fat) diet (#112280) for five weeks to induce simple steatosis (Dyets, Inc., Bethlehem, PA). Mice were placed on a methionine and choline deficient (MCD) diet (N=5) (#518810) for eight weeks to induce NASH or a methionine and choline re-supplemented diet (N=3) (#518754) as a control (Dyets, Inc., Bethlehem, PA). Due to no histopathological changes observed between both control diets (isocaloric and methionine choline re-supplemented diets) they were both combined as a single control group in statistical analyses for all experiments conducted in this study.

Arsenic Disposition Study. Following 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_2O. Dosing was performed using 20 gauge

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feeding needles (Fisher Scientific, Pittsburgh, PA). Following dosing, the mice were placed in Nalgene metabolic cages, where total urine was collected for 24h. At the conclusion of the 24h period, the mice were euthanized by CO_2 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 were snap-frozen in liquid nitrogen, and stored at -80º C until analysis. Total urine was transferred to a sterile collection tube and stored at -80º C prior to analysis.

Sample Preparation. The extraction of arsenical species from liver and kidney was carried out using previously described methods (Csanaky *et al.*, 2003). All reagents were prepared in HPLC-grade H_2O. Briefly, ~200 mg of tissue was homogenized in 1 ml of cold 0.4 M $HClO_4$. Five hundred micro liters of homogenate was transferred to a sterile collection tube containing 50 µl of aqueous 150 mM $HgCl_2$ then gently mixed and kept on ice for one minute. The samples were centrifuged at 15,850 x g for two minutes at 4ºC. The supernatant was removed and transferred to a sterile collection tube and the pH was adjusted to ~2 using 1 M KOH. Samples were centrifuged at 15,850 x g for two minutes at 4ºC and the supernatant was removed and stored at -80º C prior to analysis for arsenic speciation. It must be noted that although these samples were treated with $HClO_4$ and $HgCl_2$ 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 since all the samples were treated and processed in the same manner, it is conceivable that any difference in the pattern of

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arsenic oxidation state across the samples tested cannot be explained by $HgCl_2$ and $HClO_4$ treatment but rather the inherent differences in the tissue samples themselves.

Determination of Arsenic Species in Urine and Tissue. The arsenic speciation method is adapted from Gong, Z., *et al.*, 2001. Urine and previously extracted tissue homogenates were filtered through 0.45 µm nylon centrifuge filters and diluted prior to injection into the HPLC system. The HPLC system consisted of an Agilent 1100 HPLC (Agilent Technologies Inc., Santa Clara, CA) with a reverse-phase C18 column (Gemini 5µm C18 110Å, 150x4.60mm, Phenomenex, Torrance, CA) and guard cartridge. The mobile phase (pH 5.85) contained 4.7mM tetrabutylammonium hydroxide, 2mM malonic acid and 4% (v/v) methanol at a flow rate of 1.2 ml/min. The column temperature was maintained at 50ºC and samples were kept at 4ºC in a thermally controlled autosampler. An Agilent 7500ce inductively coupled plasma mass spectrometer (ICP-MS) with a Conikal nebulizer (Glass Expansion) was used as the detector. The operating parameters were as follows: Rf power 1500 watts, plasma gas flow 15 L/min, carrier flow ~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 ~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 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 50mL) at 4°C. Homogenized tissue was then agitated at 4°C for 2 hours, centrifuged at 10,000 x g for 30 minutes, and the supernatant transferred to a clean collection tube. Protein concentrations were determined using the Pierce BCA Protein Quantitation Assay (Thermo Scientific, Rockford, IL) per the manufacturer's protocol.

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Immunoblot Protein Analysis. Whole cell lysate proteins (75 µg/well) were prepared in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with β-mercaptoethanol and heated at 90ºC for ~7 minutes prior to separation by SDS-PAGE on 10% gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes overnight at 4ºC. Following transfer, the membranes were blocked in 5 % nonfat dry milk diluted in either phosphate-buffered saline-tween 20 (Mrp1) or tris-buffered saline-tween 20 (As3mt) for 45 minutes at room temperature. To determine relative protein levels of As3mt, a rabbit polyclonal antibody developed with full-length human As3MT and containing cross-reactivity towards both rat and mouse As3mt was used (kindly donated by Walter Klimecki at the University of Arizona, Tucson). Relative protein levels of Mrp1 was 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 4ºC with constant rocking. The following HRP-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 Image J software (NIH, Bethesda, MD) and normalized to ERK 1 protein (C-16, Santa Cruz, CA). 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 GAPDH has been shown to be inconsistent, perhaps due to the association of NASH with fibrosis.

Statistical Analysis. Data was analyzed using one-way ANOVA to determine significant findings among diet groups with a Bonferroni post-hoc analysis. A two-way ANOVA was used to determine significance between arsenic metabolites found in urine and tissue

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amongst 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 carried out using GraphPad Prism software (GraphPad Software, Inc., La Jolla, 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 Figure 1. Microvesicular lipid deposits are clearly visible in the liver of mice fed a high fat diet for five 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 previously characterized 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 24h arsenic excretion in the urine of control, steatosis, and NASH mice is shown in Figure 2. Mice with NASH excreted a significantly higher proportion ($p < 0.05$) of total arsenic in 24h compared to control following a single, oral dose of 0.2 mg/kg arsenic trioxide (Figure 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 mg/kg sodium arsenate did not excrete significantly higher levels of arsenic in their urine compared to control (Figure 2B).

A breakdown of the relative amount of arsenic metabolites excreted in 24h urine of control, steatosis, and NASH mice dosed with arsenic trioxide and sodium arsenate is shown in Figure 3. The dimethylated arsenic metabolite (DMA) 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 ($p < 0.05$) inorganic arsenic (iAs) compared to controls following arsenic trioxide treatment (Figure 3A). No difference in the relative amounts of metabolites excreted in 24h urine was detected amongst disease groups in animals treated with sodium arsenate (Figure 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 (Figure 4). Specifically, mice with NASH excreted a statistically higher ($p < 0.05$) percentage of the dose as trivalent iAs compared to control following a single, oral dose of arsenic trioxide (Figure 4B). However, urinary excretion of trivalent iAs in NASH versus control mice given sodium arsenate did not reach statistical significance. (Figure 4D). No change in the excretion of pentavalent iAs in urine was observed among disease states (Figure 4A and Figure 4C).

Renal and Hepatic Accumulation of Arsenic in Experimental NAFLD. Arsenic accumulation in the liver and kidneys of mice was evaluated 24h 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 amongst disease groups (Figure 5A and Figure 5C). In contrast, the accumulation of specific arsenic metabolites was altered in a disease-dependent fashion (Figure 5B and Figure 5D). Mice with NASH that were dosed with arsenic trioxide and sodium arsenate accumulated significantly higher ($p < 0.05$) MMA levels in liver compared to controls as well as 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 Figure 6. No significant difference in the renal levels of total arsenic was identified in the kidneys 24h post-dose for either arsenic trioxide or sodium arsenate between disease groups (Fig. 6A and Fig. 6C). However, similar to what was observed in the liver, the retention of arsenic metabolites in kidney was altered in a disease-dependent fashion (Fig. 6B and Fig. 6D). 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 measured in livers of control, steatosis and NASH mice 24h post dose 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 to control (Fig. 7A and Fig. 7C). Conversely, no statistically significant difference in the levels of trivalent iAs was observed amongst disease groups in both arsenic trioxide and sodium arsenate treated mice (Fig. 7B and Fig. 7D).

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 ($p<0.05$) downregulated in the livers of mice with steatosis compared to 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 to 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 non-malignant diseases (Thomas *et al.*, 2001;Rosen *et al.*, 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 currently 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 investigating the effects of hepatitis, steatosis, and alcoholic cirrhosis in humans have also reported an increase in the urinary elimination of arsenic compared to 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 impact the elimination of arsenic. A recent 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 As3mt and uses the methyl donor s-adenosyl methionine (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 As3mt in the liver. No significant change in protein expression of hepatic As3mt was detected in NASH animals, suggesting that the increase in total urinary excretion is not due to alterations in metabolism. These findings coincide with the observation that the relative amounts of both mono and dimethylated arsenic metabolites recovered in the urine were not altered by disease state. Interestingly, a significant downregulation 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 recent 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 compensate for the decrease in As3mt expression seen in mice with steatosis. Additionally, pre-systemic 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 *et al.*, 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 impact the methylation efficiency of arsenic in humans as well as animals (Heck *et al.*, 2009;Jin *et al.*, 2010). In addition, the effects of the MCD diet on hepatic SAM levels in mice remains controversial as some groups have reported depletion in hepatic SAM whereas others report no change in hepatic SAM after 6 weeks of MCD diet (Caballero *et al.,* 2010; Gyamfi *et al.,* 2008). 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 72h 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 as no change in total hepatic arsenic retention or urinary excretion of arsenic metabolites was observed. Interestingly, 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 (MMA^{III}) and DMA (DMA^{III}) 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). Due to 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 inorganic arsenic (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 ABCC subfamily of efflux transporters, the multidrug-resistance associated protein 1 (MRP1), has been implicated in the cellular transport of arsenic (Lorico *et al.*, 2002;Thomas, 2007). Interestingly, MRP1 has been shown to transport $arsenite^{III}$ but not $arsenate^{V}$ 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 inorganic arsenic over its pentavalent counterpart. Additionally, an MRP1 over-expressing cell line has been shown to confer resistance to MMA^{III} but not MMA^{V} in a glutathione-dependent fashion suggesting that

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MMA^{III} 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 inorganic arsenic 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-glutathione conjugates (Kala *et al.*, 2000;Carew *et al.*, 2010;Kala *et al.*, 2004). 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;Hardwick *et al*., 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, Kopplin, Lake.

Contributed new reagents: Scheffer, Klimecki

Performed data analysis: Canet, Cherrington.

Wrote or contributed to the writing of the manuscript: Canet, Cherrington.

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Footnotes

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Figure Legends

Figure 1. Liver Histopathology of Mice fed a Control, High Fat and MCD Diet. Representative hematoxylin and eosin stained liver sections from control (A), high fat (B), and MCD (C) fed mice. Microvesicular steatosis identified in the high fat fed mice 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 20x magnification.

Figure 2. Total Arsenic Elimination in Urine. Percent of total arsenic dose excreted in 24h urine from control, steatosis, and NASH mice given a single oral dose of arsenic trioxide (A), or sodium arsenate (B). Data represent the mean ± S.D. from 4-6 mice. * $p < 0.05$ versus control mice.

Figure 3. Urine Profile of Arsenic Metabolites. Relative amount of arsenic metabolites recovered in 24h urine from control, steatosis, and NASH mice given a single, oral dose of arsenic trioxide (A), or sodium arsenate (B). Data represent the mean ± S.D from 4-6 mice. * $p < 0.05$ versus control mice.

Figure 4. Differential Elimination of Trivalent and Pentavalent iAs in Urine. Percent of the arsenic dose excreted as pentavalent (A, C) and trivalent (B, D) iAs in 24h urine from control, steatosis, and NASH mice. Mice were given either a single, oral dose of arsenic trioxide (A, B), or sodium arsenate (C, D). Data represent the mean ± S.D. from 4-6 mice. * $p < 0.05$ versus control mice.

Figure 5. Liver Accumulation of Arsenic Metabolites. Total arsenic retained in the livers of control, steatosis, and NASH mice 24h post-dose given a single, oral dose of arsenic trioxide (A, B), or sodium arsenate (C, D). A and C depict total arsenic retained in liver, while B and D depict the percent of arsenic species present in the liver 24h post-dose. Data represent the mean ± S.D. from 4-6 mice. * $p < 0.05$ versus control mice.

Figure 6. Kidney Accumulation of Arsenic Metabolites. Total arsenic retained in the kidneys of control, steatosis, and NASH mice 24h post-dose given a single, oral dose of arsenic trioxide (A, B), or sodium arsenate (C, D). A and C depict total arsenic retained in kidneys. B and D depict percent of arsenic species present in the kidneys 24h post-dose. Data represent the mean ± S.D. from 4-6 mice. * $p < 0.05$ versus control mice.

Figure 7. Differential Accumulation of Arsenic Metabolites in Liver. Percent of the arsenic dose retained in the liver as pentavalent (A, C) and trivalent (B, D) iAs 24h post-arsenic dose from control, steatosis, and NASH mice. Mice were given a single, oral dose of arsenic trioxide (A, B) or sodium arsenate (C, D). Data represent the mean ± S.D. from 4-6 mice. * $p < 0.05$ versus control mice.

Figure 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 is normalized to Erk protein expression. Data represent the mean ± S.D. from 4-6 mice. * $p < 0.05$ versus control mice

Figure 1

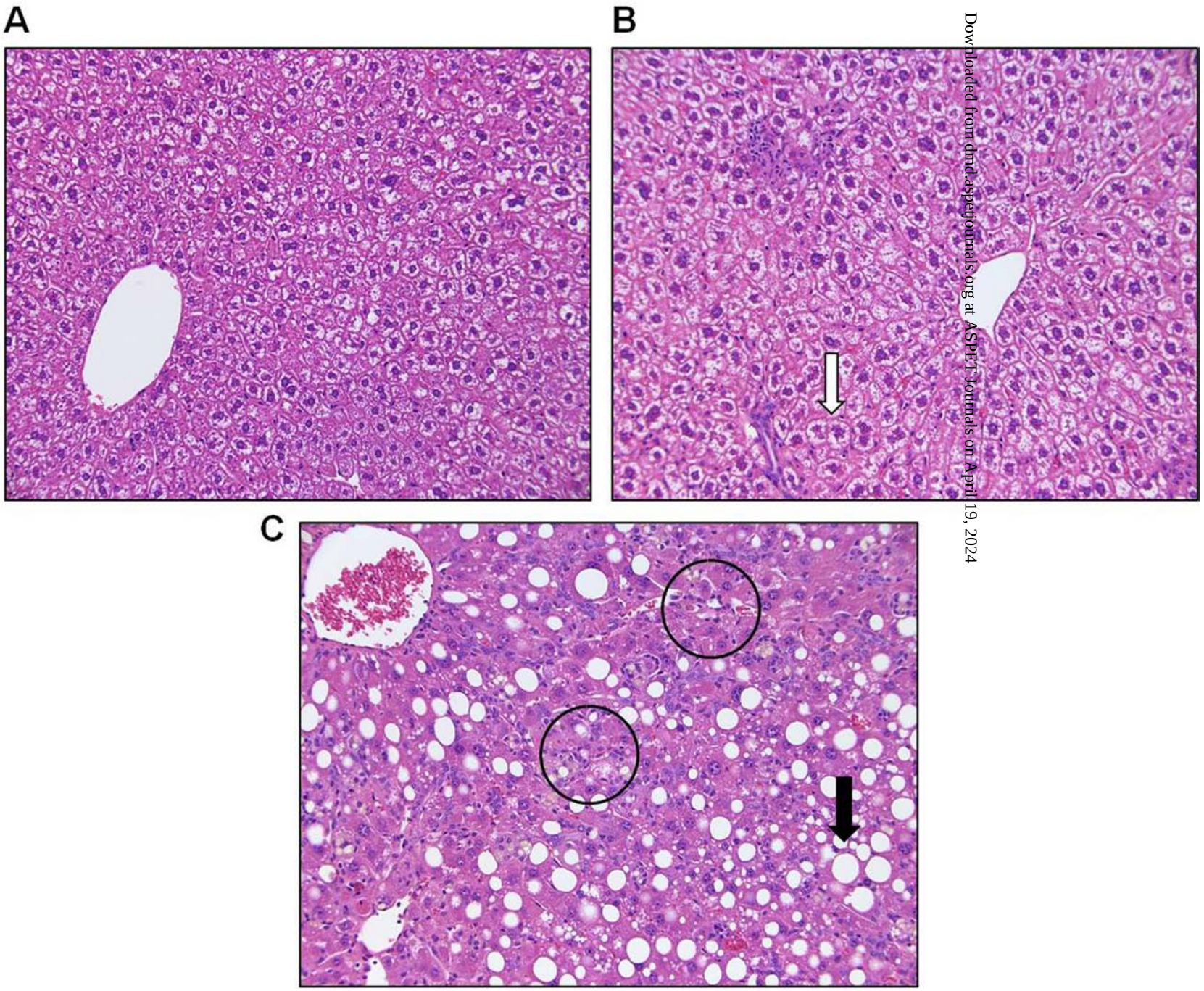
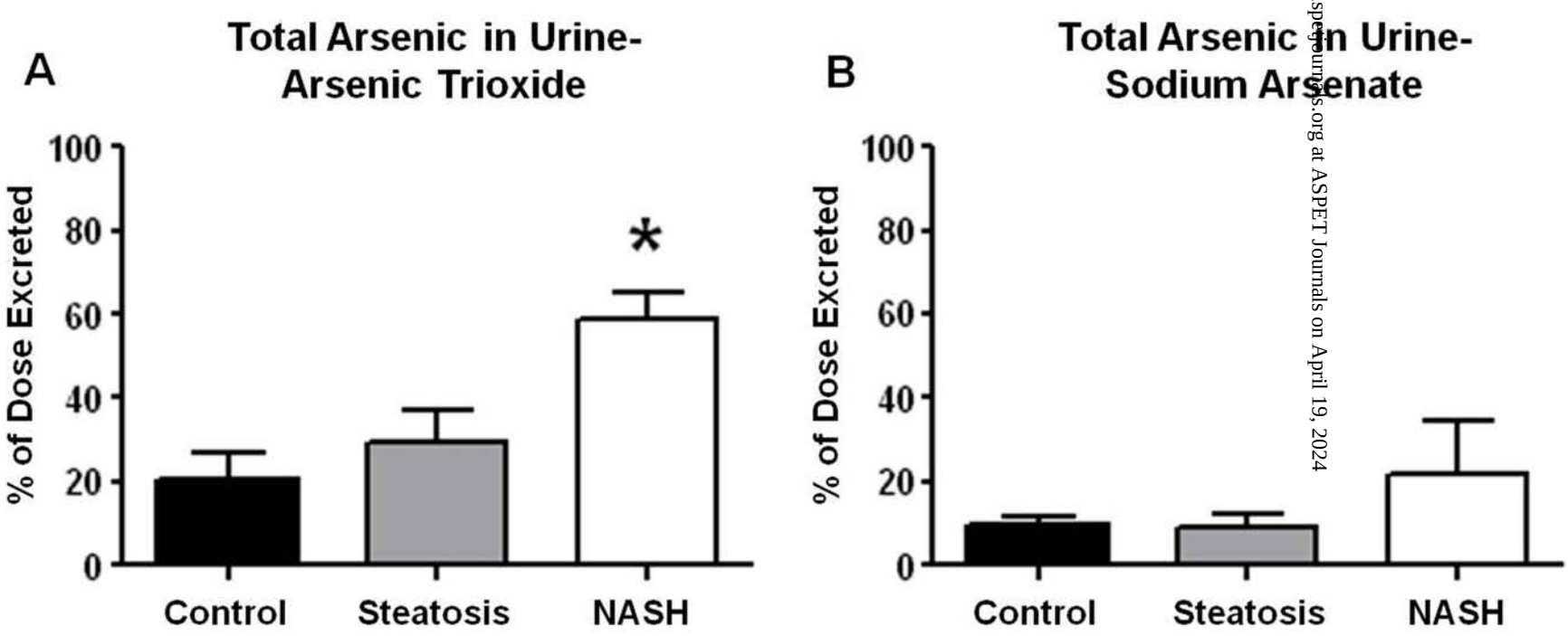


Figure 2



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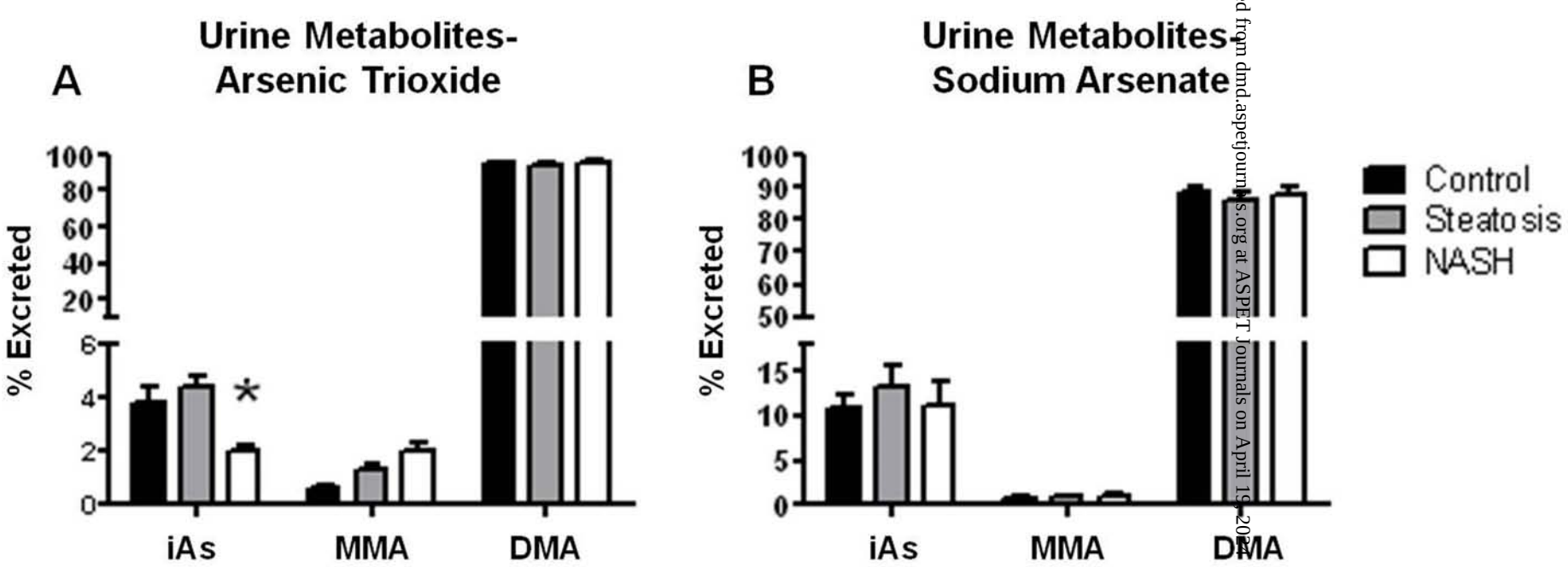

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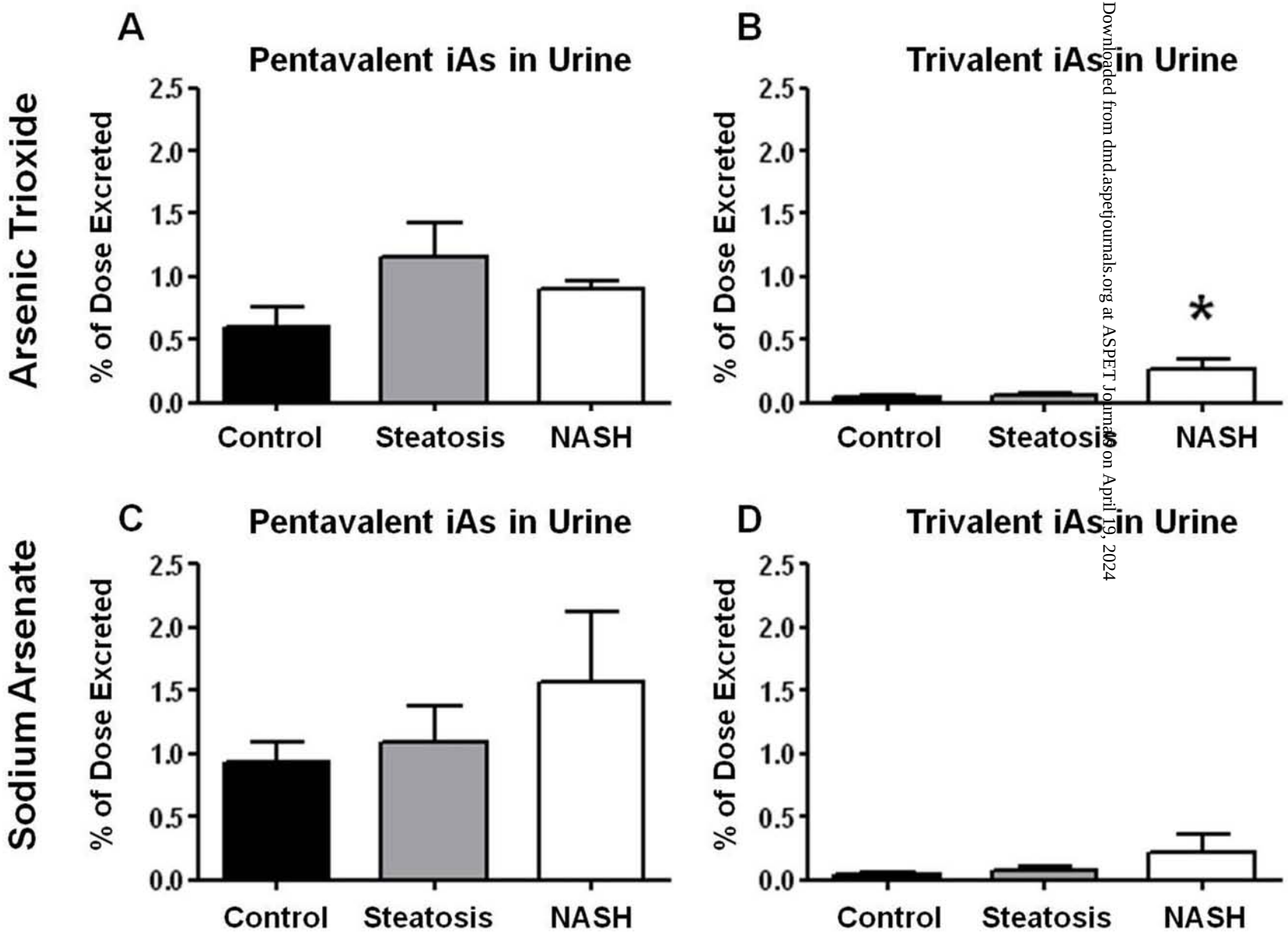

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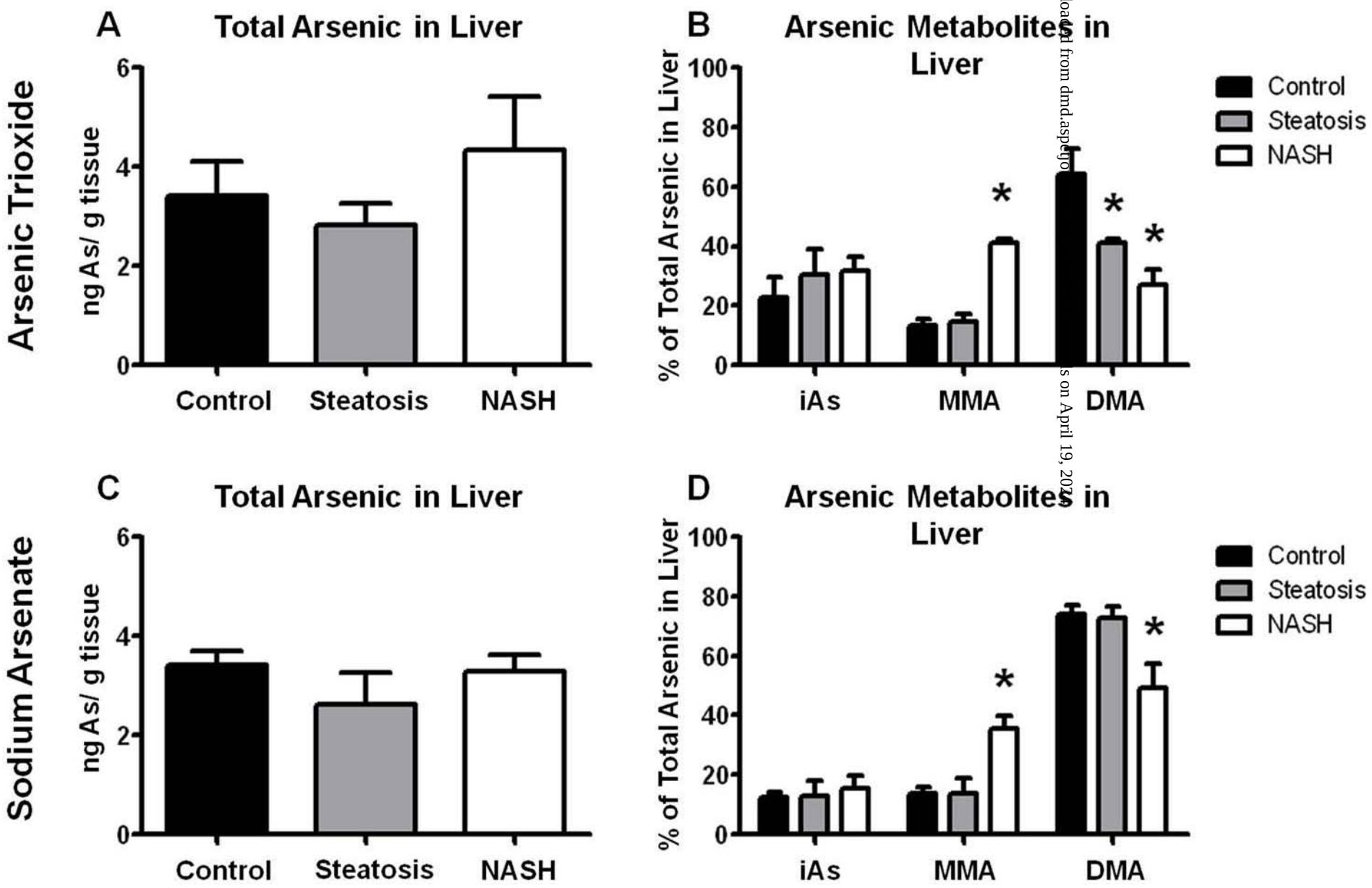

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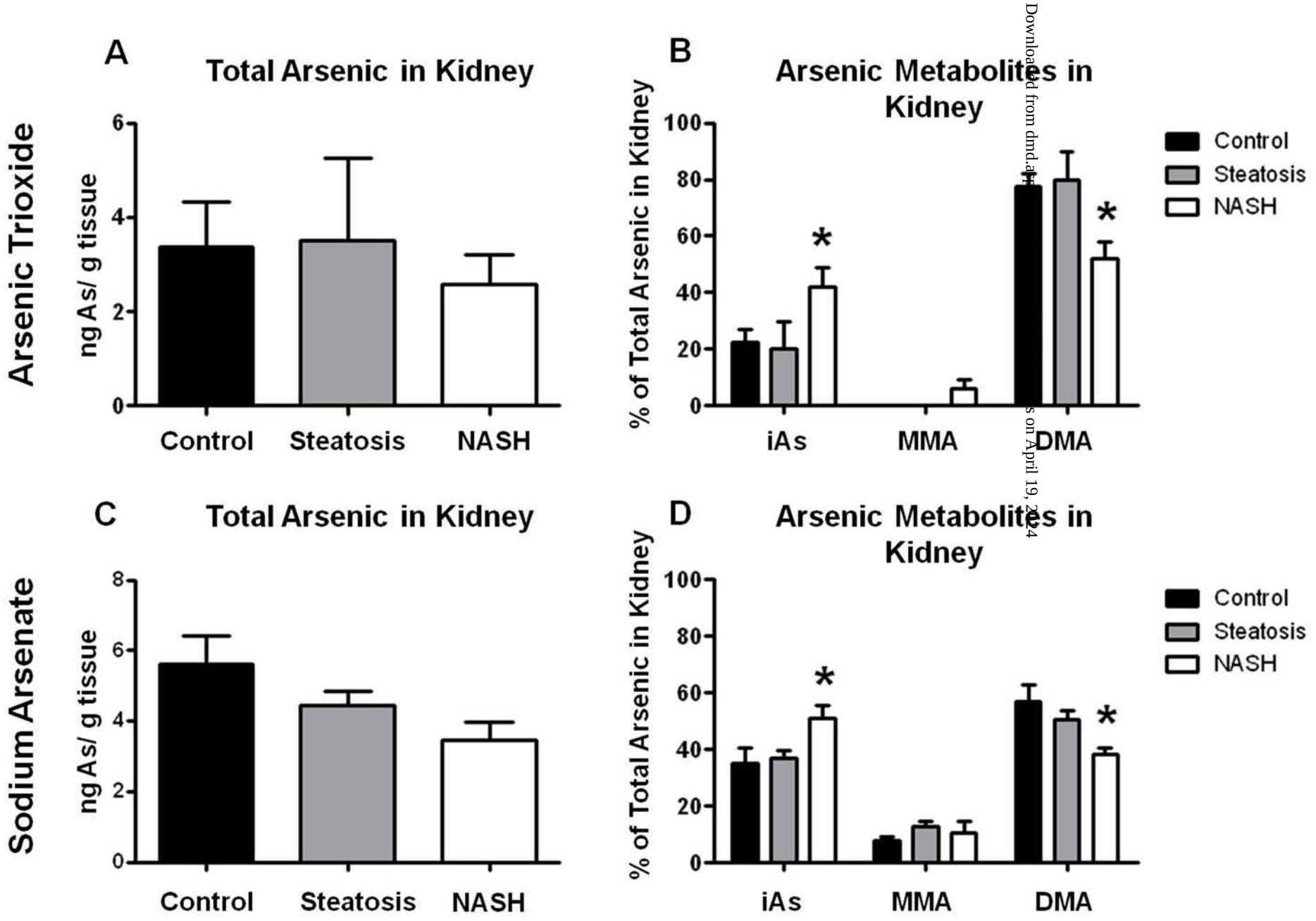

Figure 7

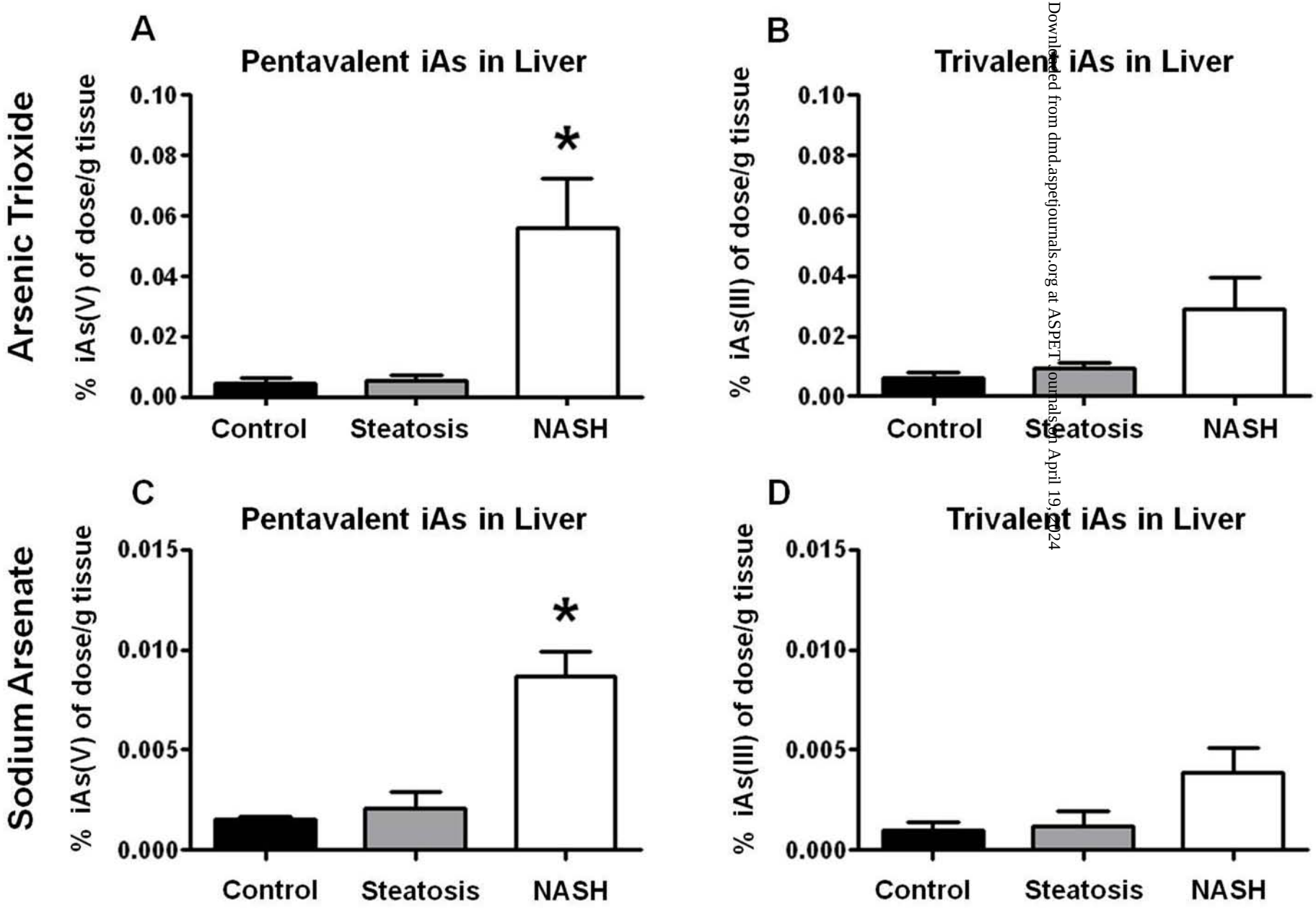

Figure 8