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Effects of Arsenite Exposure during Fetal Development on Energy Metabolism and Susceptibility to Diet-Induced Fatty Liver Disease in Male Mice

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Running title: As (III) exposure and altered energy metabolism

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Abstract

Background: Chronic exposure to arsenicals, at various life stages and across a range of exposures, has been implicated in cardiometabolic disease and liver disease, but disease predisposition from developmental exposures remains unclear.

Objectives: *In utero* and post-weaning As (III) exposure was examined on the background of a Western diet to determine whether As (III) affects metabolic disease.

Methods: Male Swiss Webster mice were exposed to 100 ppb As (III) *in utero*, after weaning, or both. *Ad libitum* access to a Western diet was provided after weaning, and the plasma metabolome, liver histopathology, liver enzyme activity, and gene expression were analyzed.

Results: Hepatic lipid composition and histopathology revealed that developmental As (III) exposure exacerbated Western diet induced fatty liver disease. Continuous As (III) exposure increased cardiometabolic risk factors including increased body weight, insulin resistance, hyperglycemia, and plasma triglycerides. As (III) affected a decrease in the intermediates of glycolysis and the TCA cycle, while increasing ketones. Hepatic isocitrate dehydrogenase activity was also decreased, which confirms disruption of the TCA cycle. Developmental As (III) increased expression of genes involved in fatty acid synthesis, lipogenesis, inflammation, and packaging of triglycerides suggesting increased Acetyl-CoA load.

Conclusions: *In utero* and continuous early life exposure to As (III) disrupted normal metabolism and elevated the risk for fatty liver disease in mice maintained on a high fat diet. Our findings suggest that individuals exposed to As (III) during key developmental periods, and who remain exposed to As (III) on the background of a Western diet, may be at increased risk of metabolic disease later in life.

Introduction

Chronic arsenic exposure has become more prevalent with a shift from sources of water on the surface to drilling of wells to reach ‘cleaner’ water. The use of well water increases the risk for individuals to be exposed to arsenic (Yoshida et al. 2004). Arsenical exposure increases mortality from cardiovascular disease and hypertension in populations exposed to arsenicals during gestation as well as into adulthood (Hawkesworth et al. 2013; Yuan et al. 2007).

Cardiometabolic syndrome is a set of metabolic dysfunctions combined with increased blood pressure that culminates in an increased risk for cardiovascular disease (Kirk and Klein 2009).

The relationship between metabolic syndrome and arsenic has been strengthened over the years, but conflicting results appear in recent epidemiological studies (Brauner et al. 2014; Chen et al. 2010). A contributing factor to this discrepancy could be variables such as regional differences in nutrition. In addition, only a few studies have focused on the impact of *in utero* and early life exposures to low-level arsenicals, and the need to investigate this type of exposure has been highlighted by the NIEHS (Davila-Esqueda et al. 2011; Maull et al. 2012; States et al. 2012).

The majority of studies investigating nonalcoholic fatty liver disease (NAFLD), from benign steatosis to end-stage liver disease in non-alcoholic steatohepatitis (NASH), have principally focused on arsenical exposures in the parts per million ranges, that while environmentally relevant in some areas, left much uncertainty about the potential effects of chronic exposures in the parts per billion range (Arteel et al. 2008; Reilly et al. 2014; Shi et al. 2014; Tan et al. 2011).

In addition, our laboratory has demonstrated incidences of NAFLD with low-level *in utero* exposure in mice, and this work aims to expand on those initial findings (Sanchez-Soria et al. 2014). Incidence of NAFLD is important to consider when examining cardiometabolic disease

because NAFLD is thought to be the hepatic manifestation of metabolic syndrome (Paschos and Paletas 2009). There is also an association with elevated mediators of atherosclerosis in patients with NAFLD suggesting a link to cardiovascular disease (Sookoian et al. 2010).

The objective of this study was to examine low-level (100 ppb) As (III) effects on mice that were exposed during gestation, after weaning, or throughout life, with all mice being exposed to a Western diet after weaning. We have previously shown that low-level As (III) exposure *in utero* was associated with incidence of fatty liver disease, so this study aimed to reproduce these results on the background of a high fat diet in order to determine whether As (III) exposure contributes to the incidence and severity of NAFLD (Sanchez-Soria et al. 2014). In addition, components of cardiometabolic syndrome were investigated in order to examine how these disease risk factors may be affected by low-level As (III) exposure.

Materials and Methods

Animals and Treatment

Primi-pregnant CFW Swiss Webster mice were purchased from Charles River Laboratories and housed individually in sterile microisolator cages (5 inches high with 75 square inches of floor) with corncob bedding (7097.25 Corncob, Harlan Laboratories Inc) on a 12 hour light/dark cycle at 20°C. This model was utilized because it has no known metabolic or cardiovascular disease predisposition that could have affected results. Food (2019 Teklad Global 19% Protein Extruded Rodent Diet, Harlan Laboratories Inc) and water were provided *ad libitum* with either 100 ppb arsenite as sodium arsenite (NaAsO₂, Sigma) or 100 ppb sodium chloride in control animals (NaCl, VWR). The Arizona Laboratory for Emerging Contaminants verified arsenite concentrations by inductively coupled plasma mass spectrometry. Control mice received 100 ppb

NaCl water; to induce *in utero* (IU) exposure, dams (53 days old) received 100 ppb NaAsO₂ water beginning at 5 days post fertilization (ED5) through birth; dams of the *in utero* and continuous (IU+) mice received 100 ppb NaAsO₂ water beginning at ED5 and pups continued on the 100 ppb NaAsO₂ water postnatal until the end of the study at 13 weeks when all animals were sacrificed via CO₂ euthanasia followed by cervical dislocation. Postnatal (PN) mice received 100 ppb NaAsO₂ water starting after weaning through the end of the study.

Out of nine total litters, six were exposed to As (III) *in utero* and three were exposed to 100 ppb NaCl. The PN and CTRL males were drawn from the three untreated litters and the six remaining *in utero* As (III) exposed litters were broken into two groups of 3 litters (IU and IU+). Litter information is included in Supplemental Material, Table S1. For the exposure groups CTRL n=10, IU n=14, IU+ n=13, and PN n=5. Litter contributions to exposure groups are included in Supplemental Material, Table S2. Water was replaced weekly. Prior to weaning, pups remained with their own mothers in one cage regardless of litter size. After weaning (21 days old), mice were provided Western diet (TestDiet) *ad libitum*, and housed with the same exposure group 4 per cage with an effort to house littermates together. This manuscript focuses solely on the male cohort.

The Western style diet utilized in this study (15.5% Protein, 40.1% Fat, 44.4% Carbohydrate by kcal with the primary ingredients being 34.05% sucrose, 19.97% milk fat, and 19.47% casein) is similar to the Standard American diet (15% Protein, 33% Fat, 50% Carbohydrate by kcal) (Grotto and Zied 2010). Body weights were measured weekly in the mornings and blood plasma was collected at weaning, 5 weeks, and 9 weeks via retro-orbital (RO) bleeding after a 6 hour fast starting at 6 am with bleeds carried out at noon. All animal use and experimental protocols

followed University of Arizona Institutional Animal Care and Use Committee (IACUC) regulations and remained in accordance with institutional guidelines ensuring that animals were treated humanely and with regard for alleviation of suffering.

Metabolomic Analysis

Plasma from week 9 RO bleeds (minimum of 7 samples per group) was subjected to metabolomic analysis (Metabolon®). A total of 337 compounds of known identity were queried with a combination of LC/MS, LC/MS/MS, and GC/MS techniques and examined for significant alterations as described previously (Ganti et al. 2012).

Histology and Pathology

Animal tissues were isolated and rinsed in cold 1x phosphate buffered saline and total liver weight was measured. The caudate liver lobe was embedded in Tissue-Tek O.C.T.TM compound (Sakura) and frozen in liquid nitrogen vapor. The median lobe was fixed in 100% ethanol, 37% formaldehyde, 100% glacial acetic acid in a 6:3:1 (v/v/v) ratio for 4 hr at RT and then overnight at 4°C with fresh fixative; both steps with slow agitation. Frozen livers were sectioned to 10 µm on a cryostat and fixed with 10% buffered formalin. The sections were stained using Oil-Red-O in propylene glycol method for lipid detection (Poly Scientific R&D Corp.). Fixed livers were processed in Paraplast 56 (McCormick Scientific), and sectioned to 10 µm. Sections were stained with hematoxylin and eosin (H&E) (Thermo Scientific) or Picrosirius Red: 0.1% Direct Red 80 (Sigma) and a saturated aqueous solution of picric acid. Oil-Red-O and H&E sections were captured using a bright field microscope and Picrosirius Red sections were captured using crossed polarizers. H&E sections were scored under blinded conditions utilizing the NAS (NAFLD activity score): the score is defined as the unweighted sum of the scores for steatosis

(0-3), lobular inflammation (0-3), and ballooning (0-2); scores 0-2 are not NASH, 3-4 are considered borderline, and 5-8 are considered NASH (Kleiner et al. 2005).

Reverse Transcription Real-Time Polymerase Chain Reaction (RT-qPCR)

RNA was isolated and purified from flash frozen tissue samples using TRIzol® according to the manufacturer's protocol (Life Technologies). First strand cDNA was generated using the Transcriptor First Strand cDNA Synthesis kit (Roche) with 1 µg RNA. Real-time Polymerase Chain Reaction (qPCR) was performed using the TaqMan Master Primer-Probe System (Roche). 40S Ribosomal Protein 7 (*Rps7*) was used as a housekeeping gene for relative quantification of target mRNA. Table 1 lists the genes of interest, the primer sequences, and the corresponding proprietary fluorescein labeled probes from Roche's Universal ProbeLibrary.

Liver Lipid Content and Enzymatic Activity Analysis

Lipids from 25 mg of flash frozen liver tissue were extracted with 500 µL of 2:1 volume ratio Chloroform and Methanol. The lipid fraction was resuspended with 1% Triton X-100 in 100% Ethanol. Triglycerides (TAG) and cholesterol were quantified using the TAG reagent set and cholesterol reagent set, respectively (Pointe Scientific). Free fatty acids (FFA) were quantified using a colorimetric free fatty acid assay kit per manufacture's protocol (Cell Biolabs). Samples from flash frozen liver tissue were analyzed with the following kits, according to the manufacturer's recommendations: Aspartate Transaminase (AST) and Isocitrate Dehydrogenase (IDH) activity assay kit (Sigma) and Alanine Transaminase (ALT) activity assay kit (Cayman Chemical).

Blood Biochemistry

Plasma samples were analyzed with the following kits, according to the manufacturer's recommendations: free fatty acid fluorometric assay kit and glucose colorimetric assay kit (Cayman Chemical), rat/mouse insulin ELISA (Millipore), and mouse hemoglobin A1c (HbA1c) assay kit (Crystal Chem). Insulin resistance was determined utilizing the homeostasis model assessment of insulin resistance (HOMA-IR) (Matthews et al. 1985).

Statistical Analysis

For all experiments, aside from metabolomics analysis (described above), a sample set with a minimum of 5 animals per treatment group in analytical duplicates were used and a one-way ANOVA followed by a Dunnett's multiple comparison test utilizing Prism6 (GraphPad) for comparisons between treatment groups and controls was performed: the reported p-value is the multiplicity adjusted p-value corrected for multiple comparisons.

Results

Effects of As (III) on Hepatic Lipids and Tissue Damage

In utero As (III) exposure increased liver weight as a proportion of body weight at 13 weeks of age, and this increase in weight was accompanied by elevated TAG in livers (Figure 1 A&B). Only mice that were continuously exposed to As (III) had increased hepatic FFA and cholesterol (Figure 1 C&D). As (III) exposed mice had increased hepatocellular ballooning degeneration, increased lipid content, and fibrosis when compared to those animals not exposed to arsenic. H&E staining of liver sections (Figure 2A-D) revealed more severe NAFLD in mice exposed to As (III) during embryonic development. Steatosis is considered more severe as it progresses outward from the centrilobular region to zone 1, and is apparent in all mice, but lipid

accumulation was more panacinar in *in utero* As (III) exposed groups (Figure 2B&C) whereas it was more limited to zones 3 and 2 in control and PN animals (Figure 2A&D). In addition, more severe hepatocellular ballooning and inflammatory lesions were observed in the *in utero* and continuously exposed As (III) exposed group. Oil Red-O Staining was used to differentiate lipids in frozen liver sections, and the most severe lipid accumulation was detected in the IU and IU+ livers (Figure 2F&G), with mild to moderate lipid accumulation occurring in the control and PN livers (Figure 2E&H). Picrosirius Red staining of liver sections under polarized light revealed no incidences of fibrosis in the livers from control mice (Figure 2I); in contrast, a number of fibrotic lesions were detected in all As (III) exposed groups (Figure 2J-L). Fibrotic lesions were generally focal and under 0.5 μm in diameter. Blinded scoring of the H&E sections (Figure 2M) showed that the NAFLD scores of the control and PN groups did not suggest NASH, the IU group had scores that were borderline, and the IU+ group had definitive NASH: the IU and IU+ groups had significantly higher NAFLD scores than control animals. Taken together, this shows the presence of NAFLD in the groups developmentally exposed to As (III) with the most severe pathology developing with continuous exposure to As (III) after birth.

As (III) Exposure and Cardiometabolic Risk Factors

IU+ exposure to As (III) resulted in an increase in cardiometabolic risk factors. IU+ mice gained more weight than controls at week 5 and remained heavier until the conclusion of the study at week 13. No statistically significant changes in weight were detected in any of the other As (III) exposure groups (Figure 3A). Insulin resistance and plasma triglyceride levels were significantly higher in IU+ mice compared to controls (Figure 3B-C). As (III) exposure did not affect plasma FFA at five weeks or blood HbA1c, a long term marker of blood glucose, at 13 weeks of age

(Data not show). Although As (III) did not affect the HbA1c or FFA concentrations, continuous As (III) exposure did increase body weight, insulin resistance, and serum triglycerides, all of which are cardiometabolic risk factors.

Effects of As (III) on Lipid and Glucose Metabolism

The previous data suggest exposure to As (III) during embryonic development primes animals for the development of cardiometabolic disease risk factors and NAFLD when exposure continues throughout life. As such, As (III) induced alterations in energy metabolism were investigated by metabolomic analysis on plasma samples of mice to identify changes that may contribute to metabolic disease. Table 2 highlights biochemicals in plasma found to be substantially altered in As (III) exposed mice when compared to controls. 1,5-anhydroglucitol was decreased only in the IU+ mice compared to controls. This depletion suggests recent (< 1 month) hyperglycemia (Dungan 2008). In addition, key intermediates and end products in glycolysis were differentially altered depending on exposure period. Intermediates within the TCA cycle were decreased primarily in *in utero* As (III) exposed mouse plasma suggesting a potential disruption of oxidative energy metabolism. Increased ketone bodies were detected in the IU and IU+ mice as shown by significant increases in both beta-hydroxybutyrate and acetoacetate. In addition, a few long chain polyunsaturated fatty acids (LCPUFA) were found to be decreased in the IU group; LCPUFA can be modulators of lipid metabolism. Taken together, these data suggest that As (III) exposure has diverse and profound effects on energy metabolism including alterations in glycolysis, the TCA cycle, and ketogenesis.

As (III) and TCA Cycle and Transaminase Enzymatic Activity

The activity of hepatic IDH was detected to confirm the attenuation of TCA cycle activity observed in the plasma metabolomic analysis. IDH is upstream of these metabolites and it is reported to be inhibited by As (III) (Higashi et al. 1965). A significant decrease in IDH activity was detected in livers from the IU and IU+ mice (Figure 4A). A decrease in hepatic AST or ALT activity is associated with fatty liver disease (Pol et al. 1991). A significant decrease in hepatic AST activity was detected in the IU+ exposed group (Figure 4B) and no change in the ALT activity. The fact that PN exposure to As (III) does not significantly decrease IDH activity suggests that portions of the TCA cycle are inhibited in adult animals that were only exposed to As (III) during fetal development. Therefore *in utero* exposure to arsenic is a substantial contributor to disruption of the normal TCA cycle in adulthood.

As (III) and the Hepatic Transcription Profile

The hepatic expression of several key genes involved in metabolism, lipid uptake, inflammation, and triglyceride export were detected in order to determine the effects of As (III) exposure on their expression and whether they contribute to disrupted lipid homeostasis (Figure 5).

Expression of Fatty acid uptake transporter *Cd36* was significantly increased only in the IU+ group. mRNA for *Pparg*, a master regulator of lipid and glucose metabolism, was increased in the IU+ group. Expression of *Acaca*, the enzyme that catalyzes the carboxylation of acetyl-CoA, was significantly increased in the IU+ and PN treatment groups. mRNA of *Cpt1a*, a key player in the β -oxidation of fatty acids, was significantly increased in the IU+ treatment group. *Dgat1*, an enzyme critical to the formation of triglycerides from Acyl-CoA and diacylglycerol, was increased in livers of the PN mice only. mRNA of *Mttp*, which plays an essential role in lipoprotein assembly, was significantly increased in the livers of all As (III) treated mice.

Expression of *Tgfb1*, a pro-fibrotic growth factor, showed a trend towards an increase in the IU+ group. mRNA for *Il6*, a cytokine implicated in NAFLD, shows a trend towards an increase in the IU group. Finally, expression of *Tnf*, a cytokine that has been well established in NAFLD, is increased in the IU+ group. These changes in gene expression in the livers from *in utero* and early life As (III) exposed groups support disrupted lipid homeostasis and increased inflammatory mediators as a consequence of fetal exposure to low-level arsenic.

Discussion

This study reveals the development of metabolic disease in mice after low-level As (III) exposure during fetal development and postnatally. Figure 6 summarizes our findings using a simplified and condensed schematic of the relationship between metabolites, metabolic pathways, and genes in addition to general markers of liver damage for the IU+ exposure. NAFLD is thought to be the hepatic manifestation of metabolic syndrome (Paschos and Paletas 2009). The detection of increased incidence of NAFLD from developmental and continuous As (III) exposure in conjunction with poor glycemic control and increased insulin resistance supports arsenic induction of metabolic disease (Table 2 and Figure 3B). However, it is also important to note that no increase in HbA1c was detected. When examined together with increased body weight and increased triglycerides, these risk factors for cardiometabolic disease were restricted to the IU+ exposure group. Although it is possible that these changes are significant in the IU+ group due to the longest period of exposure, other changes were also detected in the shorter (or pulsed) IU exposure group, but not in the postnatal exposed mice. This suggests that developmental exposure to arsenic is a primer for adult onset of disease with the most severe pathology developing with continued presence of As (III).

The concentration of As (III) in this study (100 ppb) is environmentally relevant in many areas of the United States where individuals and communities rely on non-municipal, often well-based, sources of water: in addition, areas in south-east Asia are exposed to As (III) at ppm concentrations from ground water sources (Smedley and Kinniburgh 2002). However, metabolism of arsenic in these mice was not examined, so there may be unaccounted for differences in toxicokinetics. This study did include a female cohort, however, initial observations did not detect significant changes in total body weight or proportional liver weight, therefore males were analyzed to determine whether increased liver and body weight was connected to metabolic pathology.

The links between As (III) exposure and NAFLD have been established for adult exposures at higher As (III) concentrations, but little is known about the mechanisms or the impact of low-level As (III) on fetal and early developmental periods (Arteel et al. 2008; Reilly et al. 2014; Shi et al. 2014; Tan et al. 2011). Our laboratory has previously demonstrated incidence of NAFLD with *in utero* exposures to 100 ppb As (III) in mice (Sanchez-Soria et al. 2014), and is the only known example of this phenomena to our knowledge. The present work determines a mechanistic basis for arsenic effects on metabolic dysregulation and disease.

Starting at 5 weeks of age, the IU+ group was overweight when compared to controls, and remained so through the duration of the study (Figure 3A). Differential changes in weight with As (III) exposure have been demonstrated including decreased birth weights for low-level IU exposed mice (Kozul-Horvath et al. 2012; Sanchez-Soria et al. 2014). However, it was recently reported that obese adolescents appeared to metabolize arsenicals less efficiently than their normal weight counterparts, suggesting a link between obesity and susceptibility to arsenical

toxicity (Su et al. 2012). Continuous As (III) exposure increased plasma triglyceride concentrations (Figure 3C), which is contrary to earlier studies examining *in utero* exposure to arsenic. Sanchez-Soria found a decrease in plasma TAG at 4 months in Swiss Webster mice on a regular diet with *in utero* exposure to 100 ppb As (III) (Sanchez-Soria et al. 2014). Similarly Srivastava found decreased plasma TAG at 10 weeks with 49 ppm As (III) exposure on a standard diet in ApoE^{-/-} mice (Srivastava et al. 2007). Although the exact mechanism is unclear, the continuous exposure to As (III) on a Western style diet has an additive effect to increase plasma TAG explaining differences with previous studies.

The extent of the NAFLD from developmental exposure to low-level As (III) was substantial, and appeared to be most extensive in the As (III) treatment groups with *in utero* and continuous exposure (Figure 2). The increased proportional weight, TAG, FFA, and cholesterol detected in the IU (TAG and proportional weight only) and IU+ groups are strongly indicative of a NAFLD state induced by developmental exposure to As (III) that is most severe with continued exposure (Figure 1 A-D) (Puri et al. 2007; Simonen et al. 2011). Surprisingly, we detected no increase in FFA in blood plasma, which is typically associated with fatty liver disease. We suspect that this may be due to the observed NAFLD being the result of altered energy metabolism in the liver rather than an overall increase in circulating FFA (Zhang et al. 2014). We intended to examine ALT and AST activity in plasma, but small plasma samples limited the ability to probe all biochemical targets. Hepatic transaminase activity was examined as a surrogate indicator of liver damage and decreased activity of AST was detected in the IU+ group (Figure 4B) with no change in ALT activity (data not included). Elevated AST and ALT in the plasma correlate with liver injury, while decreased hepatic AST and ALT activity has been reported in fatty liver

disease (Pol et al. 1991). Therefore the observed decrease in AST activity suggests more severe NAFLD in the IU+ group.

The metabolomic data suggests disruption of the TCA cycle as decreased levels of TCA intermediates were detected in the *in utero* As (III) exposure groups (Table 2). It is known that pyruvate dehydrogenase, α -ketoglutarate dehydrogenase (Bergquist et al. 2009), IDH (Higashi et al. 1965), and succinate dehydrogenase (Hosseini et al. 2013) are disrupted by arsenical. In this regard, hepatic IDH activity was significantly reduced in the IU and IU+ animals (Figure 4A); validating disruption of the TCA cycle from developmental As (III) exposure. Due to sample limitations, other hepatic dehydrogenases involved in the TCA cycle were not analyzed, but future studies should determine if these are targets of developmental As (III) exposure. Persistent disruption of this critical process has severe repercussions on homeostasis in humans (Rustin et al. 1997). To our knowledge the continued inhibition of these dehydrogenases after *in utero* As (III) exposure is a novel finding. It is unlikely that this continued decrease in activity is a result of direct As (III) inhibition, but is rather a disruption of developmental programming. This is supported by the lack of IDH inhibition in the PN group and the remaining activity decrease in the IU group long after As (III) is removed. This reinforces the argument that developmental exposure is a primer for adult disease, especially in the context of continuous exposure after birth.

The role of arsenic in the disruption of glycolysis by replacing phosphate in the reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been well established (Hughes 2002). The changes detected in glycolysis may be a result of GAPDH disruption; however, concentrations of As (III) shown to disrupt GAPDH are much higher than 100 ppb. It

has also been shown recently that in multiple human cell lines As (III) induces the Warburg effect. Specifically, there is an increased production of lactate and an increase in expression of glycolysis related genes with low level As (III) exposure (Zhao et al. 2013). Given these findings and that arsenicals are also potent inhibitors of pyruvate dehydrogenase, an increase in lactate was expected in As (III) treated mice (Schiller et al. 1977). However, significantly lower lactate levels were detected in the IU and IU+ groups as well as an increase in pyruvate in the PN group (Table 2). Thus, there is a significant difference between *in utero* (with or without continuous exposure) and post-weaning exposure. Increased pyruvate levels could suggest decreased pyruvate dehydrogenase activity (due to known As (III) inhibition or increased Acetyl-CoA negative feedback) or decreased lactate dehydrogenase activity, which is unlikely due to a strong association between lactate dehydrogenase activity and arsenical exposure (Liao et al. 2012). This also suggests that the decrease in lactate observed in the IU and IU+ groups is unlikely to be a result of decreased lactate dehydrogenase activity, so the mechanism behind this decrease is not readily apparent. The inhibition of pyruvate dehydrogenase by negative feedback inhibition with Acetyl-CoA is consistent with our proposed mechanism of TCA cycle disruption; however, PN exposure did not appear to substantially disrupt the TCA cycle. The mechanism behind the observed increase in pyruvate in the PN group and not the *in utero* exposed groups is unclear. Ongoing exposure to As (III) in the PN group could decrease pyruvate dehydrogenase activity, but one would expect a similar effect in the IU+ group that is not observed. With developmental exposures, it is unlikely that the alterations in enzymatic activity are a result of direct As (III) disruption, but rather some form of altered developmental programming. Follow up studies examining the effects of developmental exposure on energy metabolism would be recommended to better resolve the complex and diverse changes that appear to be occurring.

The observed decrease of select LCPUFA in IU plasma only (Table 2) is puzzling as it represents one of the few changes present in only the IU treatment group. LCPUFA, as polyunsaturated fatty acids, are capable of activating a variety of peroxisome proliferator-activated receptors (Ppar) (Grygiel-Gorniak 2014), and can result in decreased activity of Srebf1 (Yoshikawa et al. 2002). Decreased LCPUFA in the liver has been shown in patients with NAFLD, and it has been suggested that this decrease may be partially responsible for development of NAFLD by decreasing suppression of SREBF1 activity and attenuating activation of Peroxisome proliferator-activated receptor alpha (PPARA). This results in conditions where TAG and FFA synthesis are favored over FFA oxidation and TAG export (Araya et al. 2004). Although we did not examine *Ppara*, we did detect expression of *Srebf1* and *Pparg* and observed an increase in expression of *Pparg* in the IU+ group, but no statistically significant change in *Srebf1* (Figure 5). Patients with NAFLD have increases in expression of both of these genes and it is thought that they work together potentiate the lipogenic state in NAFLD (Pettinelli and Videla 2011). As (III) is also capable of decreasing *Pparg* expression and signaling resulting in disruption of adipogenesis (Wauson et al. 2002): thereby contributing to the observed pathology, however, additional investigation will be needed to validate this mechanism.

We propose that As (III) mediated disruption of the hepatic TCA cycle leads to a loss of normal Acetyl-CoA flux that results in a push towards ketogenesis as shown in the plasma metabolomic findings and the increase in expression of FFA synthesis and β -oxidation related genes (Table 2 and Figure 5). The elevated expression of *Acaca* in the IU+ and PN groups suggests increased FFA synthesis and the increase in expression of *Cpt1a* in the IU+ group suggests an increase in β -oxidation (Figure 5). However, it is important to note that the product of *Acaca*, Malonyl-CoA, is an inhibitor of *Cpt1a* which prevents β -oxidation and FFA synthesis from occurring

simultaneously (Paulson et al. 1984). We propose that expression of these enzymes are increased in order to handle increased Acetyl-CoA, in the case of *Acaca*, and that expression of *Cpt1a* is increased in order to reduce FFA induced damage in a hepatoprotective manner. It is likely that increased FFA synthesis and β -oxidation are occurring, but their temporal activity differs. Increased expression of *Acaca* in the groups with ongoing exposure suggests that this change may be dependent on the continued presence of As (III). Increased expression of *Mttp* is also detected in all As (III) treated groups but *Dgat1* is increased only in the PN group (Figure 5). This suggests increased TAG export in all As (III) treated groups but only increased TAG synthesis in the PN group, which may be due to unexamined changes downstream of transcription. We also observed increased expression of *Cd36* in the IU+ treatment group, which traditionally would suggest an increase in FFA uptake, but this is inconsistent with observed plasma FFA levels which remained unchanged; however, it has recently been shown that *Cd36* is important in the regulation of VLDL secretion in the liver which may suggest a protective role against hepatic steatosis (Nassir et al. 2013). In addition, expression of cytokines involved in NAFLD and fibrosis were examined, and in the IU+ group there was a trend towards an increase in *Tgfb1* (Hasegawa et al. 2001) and an increase in *Tnf* (Crespo et al. 2001) both of which are indicative of more severe NAFLD, oftentimes pointing to NASH. Expression of *Il6* was also examined and a trend towards an increase was observed only in the IU group which is unexplained considering the most severe pathology appears in the IU+ group and *Il6* is associated with more inflammation and fibrosis in NAFLD (Wieckowska et al. 2008). Taken together, many of these gene expression changes are consistent with NAFLD and suggest increased hepatic Acetyl-CoA.

Conclusions

In summary, *in utero* and postnatal exposure to low-level As (III) combined with a Western diet promoted NAFLD and increased risk factors of cardiometabolic disease in male Swiss Webster mice. As (III) exposure during fetal development mediates disruptions in energy metabolism. Evidence for metabolic dysfunction includes altered metabolites of glycolysis as well as a disruption of the TCA cycle due in part to decreased IDH enzymatic activity. We believe that Acetyl-CoA is likely overwhelming the liver due to an impaired TCA cycle, resulting in a push towards ketogenesis and an increase in expression of FFA synthesis, β -oxidation, and TAG synthesis and export genes.

IU+ exposure resulted in the most severe pathology and was the only group to see a substantial increase in risk factors for cardiometabolic disease including: obesity, hyperglycemia, insulin resistance, and increased plasma TAG. However, based on other metabolomic changes that were observed, the severity is not necessarily a result of a longer exposure because distinct changes in metabolic endpoints occurred when comparing *in utero* and PN cohorts: thus, the observed alterations are not simply due to a longer exposure. Many changes were only detected in the IU or IU+ exposure groups and not the PN mice suggesting that fetal developmental exposure to As (III) provides unique alterations to energy metabolism that result in susceptibility to disease later in life that is most severe with continuous exposure to As (III) after birth.

References

Araya J, Rodrigo R, Videla LA, Thielemann L, Orellana M, Pettinelli P et al. 2004. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci (Lond)* 106(6):635-643; doi: 10.1042/CS20030326 [doi].

Arteel GE, Guo L, Schlierf T, Beier JI, Kaiser JP, Chen TS et al. 2008. Subhepatotoxic exposure to arsenic enhances lipopolysaccharide-induced liver injury in mice. *Toxicol Appl Pharmacol* 226(2):128-139; doi: S0041-008X(07)00381-X [pii].

Bergquist ER, Fischer RJ, Sugden KD, Martin BD. 2009. Inhibition by methylated organo-arsenicals of the respiratory 2-oxo-acid dehydrogenases. *J Organomet Chem* 694(6):973-980; doi: 10.1016/j.jorganchem.2008.12.028 [doi].

Brauner EV, Nordsborg RB, Andersen ZJ, Tjønneland A, Loft S, Raaschou-Nielsen O. 2014. Long-term exposure to low-level arsenic in drinking water and diabetes incidence: A prospective study of the diet, cancer and health cohort. *Environ Health Perspect*; doi: 10.1289/ehp.1408198 [doi].

Chen Y, Ahsan H, Slavkovich V, Peltier GL, Gluskin RT, Parvez F et al. 2010. No association between arsenic exposure from drinking water and diabetes mellitus: A cross-sectional study in bangladesh. *Environ Health Perspect* 118(9):1299-1305; doi: 10.1289/ehp.0901559; 10.1289/ehp.0901559.

Crespo J, Cayon A, Fernandez-Gil P, Hernandez-Guerra M, Mayorga M, Dominguez-Diez A et al. 2001. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* 34(6):1158-1163; doi: S0270913901088929 [pii].

Davila-Esqueda ME, Morales JM, Jimenez-Capdeville ME, De la Cruz E, Falcon-Escobedo R, Chi-Ahumada E et al. 2011. Low-level subchronic arsenic exposure from prenatal developmental stages to adult life results in an impaired glucose homeostasis. *Exp Clin Endocrinol Diabetes* 119(10):613-617; doi: 10.1055/s-0031-1287782 [doi].

Dungan KM. 2008. 1,5-anhydroglucitol (GlycoMark) as a marker of short-term glycemic control and glycemic excursions. *Expert Rev Mol Diagn* 8(1):9-19; doi: 10.1586/14737159.8.1.9 [doi].

Ganti S, Taylor SL, Abu Aboud O, Yang J, Evans C, Osier MV et al. 2012. Kidney tumor biomarkers revealed by simultaneous multiple matrix metabolomics analysis. *Cancer Res* 72(14):3471-3479; doi: 10.1158/0008-5472.CAN-11-3105 [doi].

Grotto D, Zied E. 2010. The standard american diet and its relationship to the health status of americans. *Nutr Clin Pract* 25(6):603-612; doi: 10.1177/0884533610386234 [doi].

Grygiel-Gorniak B. 2014. Peroxisome proliferator-activated receptors and their ligands: Nutritional and clinical implications--a review. *Nutr J* 13:17-2891-13-17; doi: 10.1186/1475-2891-13-17 [doi].

Hasegawa T, Yoneda M, Nakamura K, Makino I, Terano A. 2001. Plasma transforming growth factor-beta1 level and efficacy of alpha-tocopherol in patients with non-alcoholic steatohepatitis: A pilot study. *Aliment Pharmacol Ther* 15(10):1667-1672; doi: apt1083 [pii].

Hawkesworth S, Wagatsuma Y, Kippler M, Fulford AJ, Arifeen SE, Persson LA et al. 2013. Early exposure to toxic metals has a limited effect on blood pressure or kidney function in later childhood, rural bangladesh. *Int J Epidemiol* 42(1):176-185; doi: 10.1093/ije/dys215; 10.1093/ije/dys215.

Higashi T, Maruyama E, Otani T, Sakamoto Y. 1965. Studies on the isocitrate dehydrogenase. I. some properties of isocitrate dehydrogenase of beef heart muscle. *J Biochem* 57(6):793-798.

Hosseini MJ, Shaki F, Ghazi-Khansari M, Pourahmad J. 2013. Toxicity of arsenic (III) on isolated liver mitochondria: A new mechanistic approach. *Iran J Pharm Res* 12(Suppl):121-138.

Hughes MF. 2002. Arsenic toxicity and potential mechanisms of action. *Toxicol Lett* 133(1):1-16; doi: [http://dx.doi.org/10.1016/S0378-4274\(02\)00084-X](http://dx.doi.org/10.1016/S0378-4274(02)00084-X).

Kirk EP, Klein S. 2009. Pathogenesis and pathophysiology of the cardiometabolic syndrome. *J Clin Hypertens (Greenwich)* 11(12):761-765; doi: 10.1111/j.1559-4572.2009.00054.x; 10.1111/j.1559-4572.2009.00054.x.

Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW et al. 2005. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 41(6):1313-1321; doi: 10.1002/hep.20701 [doi].

Kozul-Horvath CD, Zandbergen F, Jackson BP, Enelow RI, Hamilton JW. 2012. Effects of low-dose drinking water arsenic on mouse fetal and postnatal growth and development. *PLoS One* 7(5):e38249; doi: 10.1371/journal.pone.0038249 [doi].

Liao YT, Chen CJ, Li WF, Hsu LI, Tsai LY, Huang YL et al. 2012. Elevated lactate dehydrogenase activity and increased cardiovascular mortality in the arsenic-endemic areas of southwestern taiwan. *Toxicol Appl Pharmacol* 262(3):232-237; doi: 10.1016/j.taap.2012.04.028 [doi].

Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. 1985. Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28(7):412-419.

Maul EA, Ahsan H, Edwards J, Longnecker MP, Navas-Acien A, Pi J et al. 2012. Evaluation of the association between arsenic and diabetes: A national toxicology program workshop review. *Environ Health Perspect* 120(12):1658-1670; doi: 10.1289/ehp.1104579; 10.1289/ehp.1104579.

Nassir F, Adewole OL, Brunt EM, Abumrad NA. 2013. CD36 deletion reduces VLDL secretion, modulates liver prostaglandins, and exacerbates hepatic steatosis in ob/ob mice. *J Lipid Res* 54(11):2988-2997; doi: 10.1194/jlr.M037812 [doi].

Paschos P, Paletas K. 2009. Non alcoholic fatty liver disease and metabolic syndrome. *Hippokratia* 13(1):9-19.

Paulson DJ, Ward KM, Shug AL. 1984. Malonyl CoA inhibition of carnitine palmyltransferase in rat heart mitochondria. *FEBS Lett* 176(2):381-384; doi: 0014-5793(84)81201-6 [pii].

Pettinelli P, Videla LA. 2011. Up-regulation of PPAR-gamma mRNA expression in the liver of obese patients: An additional reinforcing lipogenic mechanism to SREBP-1c induction. *J Clin Endocrinol Metab* 96(5):1424-1430; doi: 10.1210/jc.2010-2129 [doi].

Pol S, Nalpas B, Vassault A, Bousquet-Lemerrier B, Franco D, Lacour B et al. 1991. Hepatic activity and mRNA expression of aspartate aminotransferase isoenzymes in alcoholic and nonalcoholic liver disease. *Hepatology* 14(4 Pt 1):620-625; doi: S027091399100246X [pii].

Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O et al. 2007. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 46(4):1081-1090; doi: 10.1002/hep.21763 [doi].

Reilly MP, Saca JC, Hamilton A, Solano RF, Rivera JR, Whitehouse-Innis W et al. 2014. Prepubertal exposure to arsenic(III) suppresses circulating insulin-like growth factor-1 (IGF-1) delaying sexual maturation in female rats. *Reprod Toxicol* 44:41-49; doi: 10.1016/j.reprotox.2013.09.005 [doi].

Rustin P, Bourgeron T, Parfait B, Chretien D, Munnich A, Rotig A. 1997. Inborn errors of the krebs cycle: A group of unusual mitochondrial diseases in human. *Biochim Biophys Acta* 1361(2):185-197; doi: S0925-4439(97)00035-5 [pii].

Sanchez-Soria P, Broka D, Quach S, Hardwick RN, Cherrington NJ, Camenisch TD. 2014. Fetal exposure to arsenic results in hyperglycemia, hypercholesterolemia, and nonalcoholic fatty liver disease in adult mice. *Journal of Toxicology and Health* 1(1); doi: 10.7243/2056-3779-1-1.

Schiller CM, Fowler BA, Woods JS. 1977. Effects of arsenic on pyruvate dehydrogenase activation. *Environ Health Perspect* 19:205-207.

Shi X, Wei X, Koo I, Schmidt RH, Yin X, Kim SH et al. 2014. Metabolomic analysis of the effects of chronic arsenic exposure in a mouse model of diet-induced fatty liver disease. *J Proteome Res* 13(2):547-554; doi: 10.1021/pr400719u [doi].

Simonen P, Kotronen A, Hallikainen M, Sevastianova K, Makkonen J, Hakkarainen A et al. 2011. Cholesterol synthesis is increased and absorption decreased in non-alcoholic fatty liver disease independent of obesity. *J Hepatol* 54(1):153-159; doi: 10.1016/j.jhep.2010.05.037 [doi].

Smedley PL, Kinniburgh DG. 2002. A review of the source, behaviour and distribution of arsenic in natural waters. *Appl Geochem* 17(5):517-568; doi: [http://dx.doi.org/10.1016/S0883-2927\(02\)00018-5](http://dx.doi.org/10.1016/S0883-2927(02)00018-5).

Sookoian S, Castano GO, Burgueno AL, Rosselli MS, Gianotti TF, Mallardi P et al. 2010. Circulating levels and hepatic expression of molecular mediators of atherosclerosis in nonalcoholic fatty liver disease. *Atherosclerosis* 209(2):585-591; doi: 10.1016/j.atherosclerosis.2009.10.011 [doi].

Srivastava S, D'Souza SE, Sen U, States JC. 2007. In utero arsenic exposure induces early onset of atherosclerosis in ApoE^{-/-} mice. *Reprod Toxicol* 23(3):449-456; doi: 10.1016/j.reprotox.2007.01.005.

States JC, Singh AV, Knudsen TB, Rouchka EC, Ngalame NO, Arteel GE et al. 2012. Prenatal arsenic exposure alters gene expression in the adult liver to a proinflammatory state contributing

to accelerated atherosclerosis. PLoS One 7(6):e38713; doi: 10.1371/journal.pone.0038713;
10.1371/journal.pone.0038713.

Su C, Lin H, Choy C, Huang Y, Huang S, Hsueh Y. 2012. The relationship between obesity, insulin and arsenic methylation capability in taiwan adolescents. Sci Total Environ 414(0):152-158; doi: <http://dx.doi.org/10.1016/j.scitotenv.2011.10.023>.

Tan M, Schmidt RH, Beier JI, Watson WH, Zhong H, States JC et al. 2011. Chronic subhepatotoxic exposure to arsenic enhances hepatic injury caused by high fat diet in mice. Toxicol Appl Pharmacol 257(3):356-364; doi: 10.1016/j.taap.2011.09.019 [doi].

Wauson EM, Langan AS, Vorce RL. 2002. Sodium arsenite inhibits and reverses expression of adipogenic and fat cell-specific genes during in vitro adipogenesis. Toxicol Sci 65(2):211-219.

Wieckowska A, Papouchado BG, Li Z, Lopez R, Zein NN, Feldstein AE. 2008. Increased hepatic and circulating interleukin-6 levels in human nonalcoholic steatohepatitis. Am J Gastroenterol 103(6):1372-1379; doi: 10.1111/j.1572-0241.2007.01774.x [doi].

Yoshida T, Yamauchi H, Fan Sun G. 2004. Chronic health effects in people exposed to arsenic via the drinking water: Dose-response relationships in review. Toxicol Appl Pharmacol 198(3):243-252; doi: 10.1016/j.taap.2003.10.022.

Yoshikawa T, Shimano H, Yahagi N, Ide T, Amemiya-Kudo M, Matsuzaka T et al. 2002. Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. J Biol Chem 277(3):1705-1711; doi: 10.1074/jbc.M105711200 [doi].

Yuan Y, Marshall G, Ferreccio C, Steinmaus C, Selvin S, Liaw J et al. 2007. Acute myocardial infarction mortality in comparison with lung and bladder cancer mortality in arsenic-exposed region II of Chile from 1950 to 2000. *Am J Epidemiol* 166(12):1381-1391; doi: 10.1093/aje/kwm238.

Zhang J, Zhao Y, Xu C, Hong Y, Lu H, Wu J et al. 2014. Association between serum free fatty acid levels and nonalcoholic fatty liver disease: A cross-sectional study. *Sci Rep* 4:5832; doi: 10.1038/srep05832 [doi].

Zhao F, Severson P, Pacheco S, Futscher BW, Klimecki WT. 2013. Arsenic exposure induces the Warburg effect in cultured human cells. *Toxicol Appl Pharmacol* 271(1):72-77; doi: 10.1016/j.taap.2013.04.020 [doi].

Table 1. Primers and Probes for RT-qPCR.

Gene	Left (5'-3')	Right (5'-3')	Probe	NCBI RefSeq
Acaca	GGCTCAAAGTGCAGGTATCC	TTGCCAATCCACTCGAAGA	103	NM_133360.2
Apob	GAGAAGTTCGCTGCTTCCAA	CAGCAGTGCACCTTTGCGTAG	42	NM_009693.2
Cd36	TTGTACCTATACTGTGGCTAAATGAG A	CTTGTGTTTTGAACATTTCTGCTT	9	L23108.1
Cpt1a	GACTCCGCTCGCTCATT	TCTGCCATCTTGAGTGGTGA	70	NM_013495.2
Dgat1	TCGTGGTATCCTGAATTGGTG	AGGTTCTCTAAAAATAACCTTGCAT T	9	NM_010046.2
Il6	GCTACCAAAGTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA	6	NM_031168.1
Mttp	GCCCAACGTAAGTCTAATTTATGG	TGCTGGCCAACACGTCTA	55	NM_001163457. 1
Pparg	TGCTGTTATGGGTGAAACTCTG	CTGTGTCAACCATGGTAATTTCTT	2	NM_011146.3
Ppargc1 a	GAAAGGGCCAAACAGAGAGA	GTAATCACACGGCGCTCTT	29	NM_008904.2
Rps7	AGCACGTGGTCTTCATTGCT	CTGTCAGGGTACGGCTTCTG	101	NM_011300.3
Slc27a2	GCGTGCCTCAACTACAACATT	CCTCCTCCACAGCTTCTTGT	84	NM_011978.2
Srebf1	GGTTTTGAACGACATCGAAGA	CGGGAAGTCACTGTCTTGGT	78	NM_011480.3
Tgfb1	TGGAGCAACATGTGGAAGTC	GTCAGCAGCCGGTTACCA	72	NM_011577.1
Tnf	CTGTAGCCCACGTCGTAGC	TTGAGATCCATGCCGTTG	25	NM_013693.2

The examined genes are: Acetyl-CoA carboxylase 1 (*Acaca*), Apolipoprotein B-100 (*Apob*), Platelet glycoprotein 4 (*Cd36*), Carnitine O-palmitoyltransferase 1, liver isoform (*Cpt1a*), Diacylglycerol O-acyltransferase 1 (*Dgat1*), Interleukin-6 (*Il6*), microsomal triglyceride transfer protein large subunit (*Mttp*), Peroxisome proliferator-activated receptor gamma (*Pparg*), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*), 40S ribosomal protein S7 (*Rps7*), Very long-chain acyl-CoA synthetase (*Slc27a2*), Sterol regulatory element-binding protein 1 (*Srebf1*), Transforming growth factor beta-1 (*Tgfb1*), and Tumor necrosis factor (*Tnf*).

Table 2. Effects of As (III) Exposure on the Energy Metabolism Profile.

Metabolite	IU	IU+	PN	CTRL	IU	IU+	PN
1,5-anhydroglucitol	0.97	0.61*	0.99	1.08 ± 0.29	1.04 ± 0.34	0.66 ± 0.40	1.07 ± 0.34
3-phosphoglycerate	0.62†	0.34*	0.4*	2.32 ± 1.14	1.44 ± 1.10	0.79 ± 0.27	0.92 ± 0.42
2,3-diphosphoglycerate	0.51	0.29†	0.2*	3.96 ± 3.44	2.03 ± 2.43	1.16 ± 1.46	0.79 ± 0.64
phosphoenolpyruvate (PEP)	0.42*	0.28*	0.17*	3.87 ± 3.06	1.62 ± 3.09	1.10 ± 1.50	0.65 ± 0.33
pyruvate	0.81	1.19	1.64*	0.93 ± 0.40	0.76 ± 0.20	1.12 ± 0.54	1.54 ± 0.42
lactate	0.67*	0.74*	1.01	1.30 ± 0.28	0.87 ± 0.12	0.96 ± 0.20	1.32 ± 0.47
succinate	0.64†	0.86	1	1.15 ± 0.39	0.74 ± 0.14	0.99 ± 0.34	1.15 ± 0.45
fumarate	0.71†	0.61*	0.61*	1.39 ± 0.42	0.99 ± 0.35	0.85 ± 0.30	0.84 ± 0.25
malate	0.45*	0.55*	0.71	1.70 ± 0.66	0.77 ± 0.23	0.93 ± 0.53	1.21 ± 0.58
β-hydroxybutyrate	5.07*	5.5*	2.06	0.42 ± 0.14	2.11 ± 1.85	2.29 ± 1.03	0.86 ± 0.95
acetoacetate	4.81*	4.88*	1.56	0.56 ± 0.37	2.68 ± 1.67	2.72 ± 1.52	0.87 ± 0.79
dihomo-linoleate (20:2n6)	0.65*	0.88	1.05	1.09 ± 0.32	0.71 ± 0.16	0.96 ± .27	1.14 ± 0.35
mead acid (20:3n9)	0.67*	0.97	1.18	1.17 ± 0.37	0.78 ± 0.17	1.13 ± 0.42	1.38 ± 0.35
docosadienoate (22:2n6)	0.65*	0.83	0.94	1.12 ± 0.31	0.73 ± 0.22	0.93 ± 0.25	1.05 ± 0.19

Plasma metabolomic analysis shows select metabolites (with a focus on energy metabolism) found to be statistically significantly altered with As (III) treatment when compared to controls. Columns 2-4 are the fold change in metabolite compared to controls and columns 5-8 are the mean and standard deviation of the detected metabolites.(IU and IU+ n=8; CTRL and PN n=7: p ≤ 0.05 = *; p ≤ 0.1 = † compared to controls)

Figure Legends

Figure 1. Effects of As (III) Exposure on Liver Weight and Hepatic Lipid Content. (A) Increased proportional liver weight with As (III) treatment at sacrifice. (B) Increased triglycerides in IU and IU+ livers. (C) Increased cholesterol and (D) increased free fatty acids in IU+ livers only. The box extends from the 25th to the 75th percentile and whiskers show the entire distribution: the line is the median and the + is the mean. ((A) CTRL n=10, IU n=14, IU+ n=13, PN n=5; (B&C) CTRL n=9, IU n=14, IU+ n=6, PN n=5; (D) CTRL, IU, and IU+ n=6, PN n=5: $p \leq 0.0001 = ****$; $p \leq 0.01 = **$; $p \leq 0.05 = *$; $p \leq 0.1 = \dagger$ compared to controls)

Figure 2. Effects of As (III) on Lipid Accumulation, Hepatocellular Damage, and Fibrosis. Rows represent each different stain utilized on sections made at sacrifice, and columns contain a representative section from each treatment group. The first row, H&E staining (0.3 mm bar for scale), demonstrates various degrees and distribution of steatosis, inflammation, and hepatocellular ballooning. In the second row, Oil-Red-O (0.2 mm bar for scale), lipids stain red demonstrating increased lipid accumulation across As (III) treatment groups. In the third row, Picrosirius Red (0.15 mm bar for scale), white arrows highlight birefringence specific for collagen indicating fibrosis. The NAS scoring of the H&E sections (M) shows little to no NASH in the CTRL and PN groups, equivocal NASH in the IU group, and definitive NASH in the IU+ group. The box extends from the 25th to the 75th percentile and whiskers show the entire distribution: the line is the median and the + is the mean. ((A-M) CTRL n=6, IU n=7, IU+ n=8, PN n=5: $p \leq 0.001 = ***$; $p \leq 0.05 = *$ compared to controls)

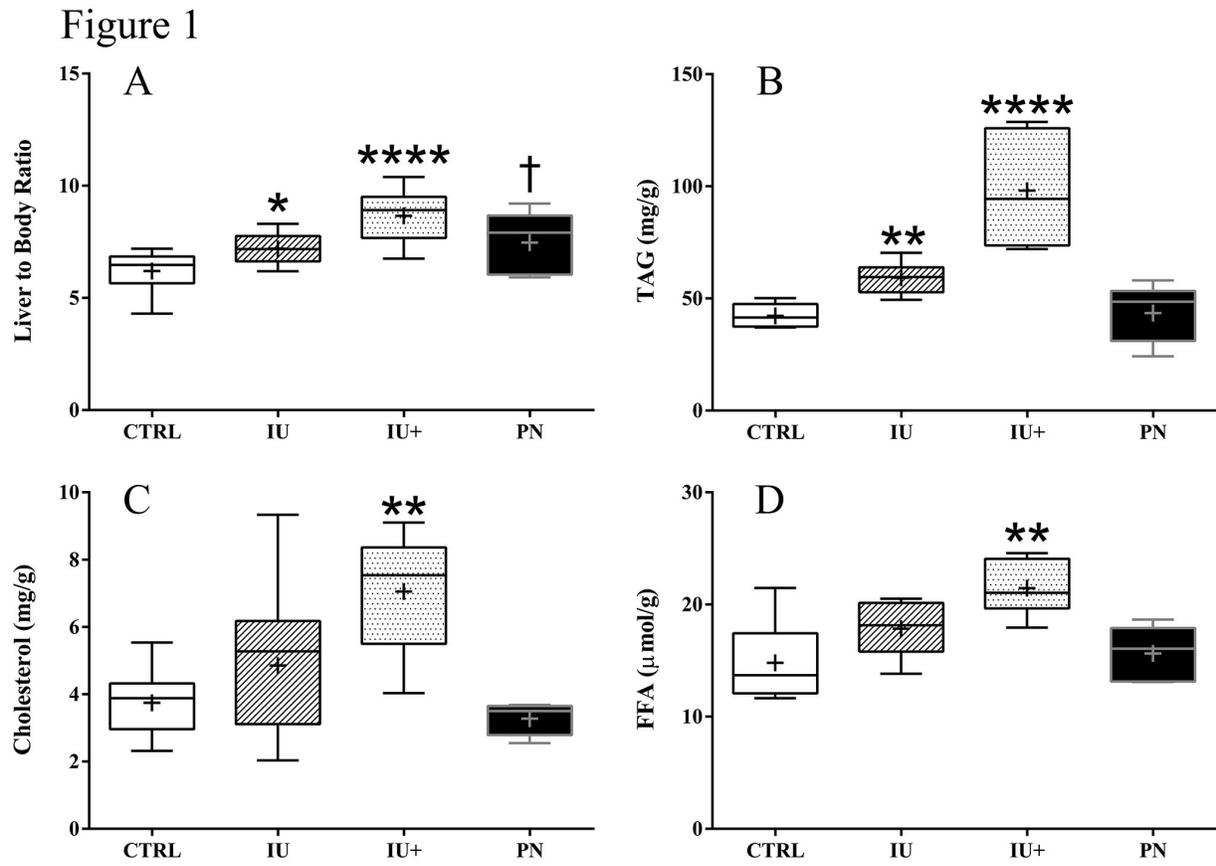
Figure 3. As (III) Exposure and Cardiometabolic Risk Factors. IU+ treatment group found to have (A) increased body weight 5 weeks after birth, (B) increased insulin resistance at week 5, and (C) increased circulating triglycerides at week 5. Error bars in (A) represent standard error. The box extends from 25th to 75th percentile and whiskers show the entire distribution: the line is the median and the + is the mean. ((A) At 13 weeks CTRL n= 10, IU n= 14, IU+ n=13, PN n=5; (B&C) CTRL n= 10, IU and IU+ n=11, PN n=7: $p \leq 0.001 = ***$; $p \leq 0.01 = **$; $p \leq 0.05 = *$ compared to controls)

Figure 4. As (III) Exposure and TCA Cycle and AST Enzymatic Activity. (A) IDH activity is decreased in the livers of IU and IU+ treatment groups, but (B) AST activity is only decreased

in the IU+ group. The box extends from the 25th to the 75th percentile and whiskers show the entire distribution: the line is the median and the + is the mean. ((A) CTRL, IU+, and PN n=5; IU n=6; (B) CTRL, IU, and IU+ n=6; PN n=5: $p \leq 0.0001 = ****$; $p \leq 0.05 = *$ compared to controls)

Figure 5. As (III) Exposure and the Hepatic Transcription Profile. mRNA levels of lipid handling and cytokine genes are shown as fold change compared to controls, and error bars represent standard error. (n=5: $p \leq 0.05 = *$; $p \leq 0.01 = **$; $p \leq 0.1 = \dagger$ compared to controls)

Figure 6. Schematic Summary of the Detected Changes from Developmental Exposure to As (III). This is an abbreviated list of alterations observed in the IU+ group with a schematic representation of their relationships to each other. (\uparrow = an increase compared to controls, \downarrow = a decrease compared to controls, $|$ = no change compared to controls, $*$ = data from week 9 blood plasma metabolomics, $\#$ = data from week 5 blood plasma, and \dagger = hepatic RT-qPCR data)



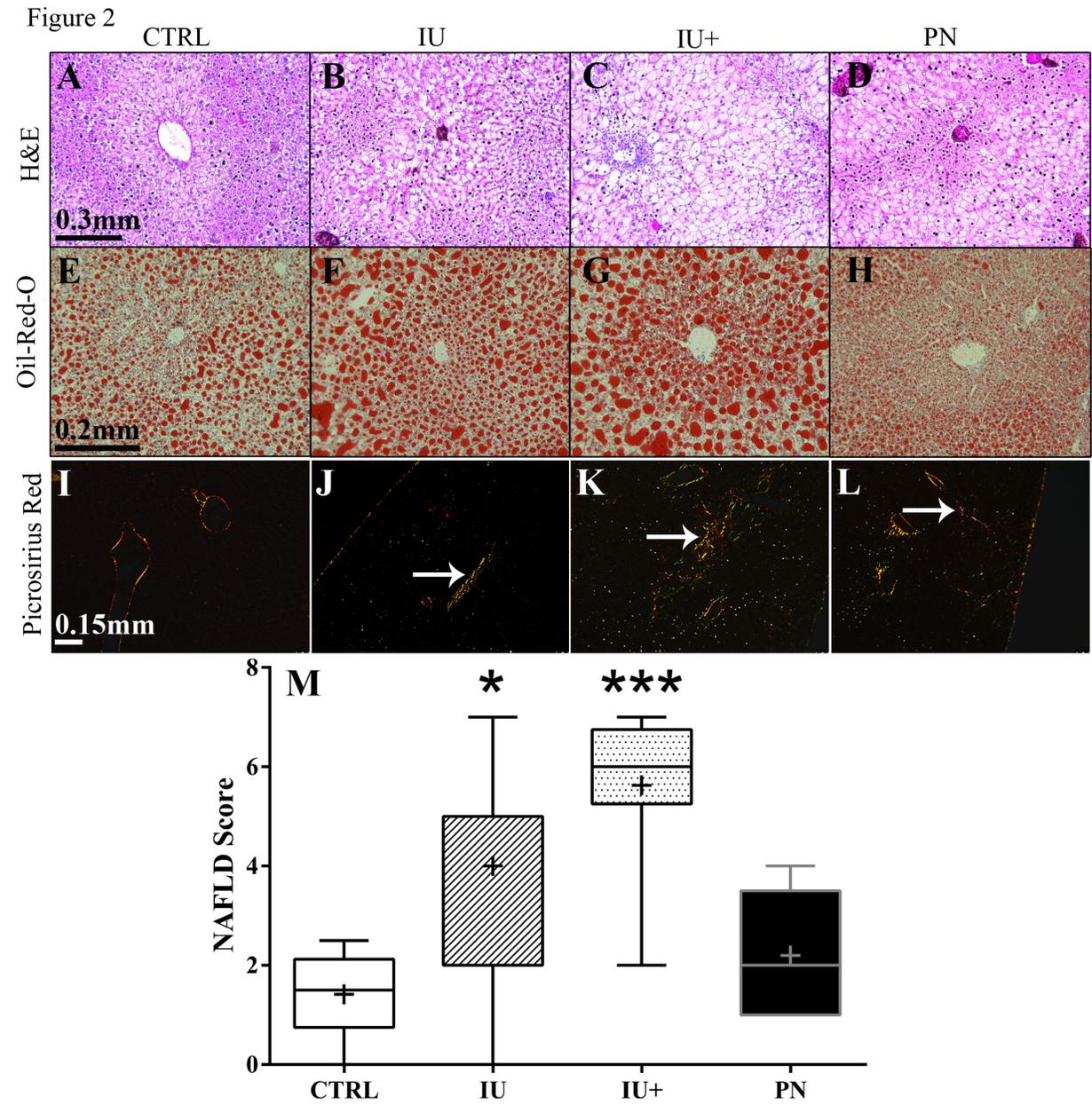


Figure 3

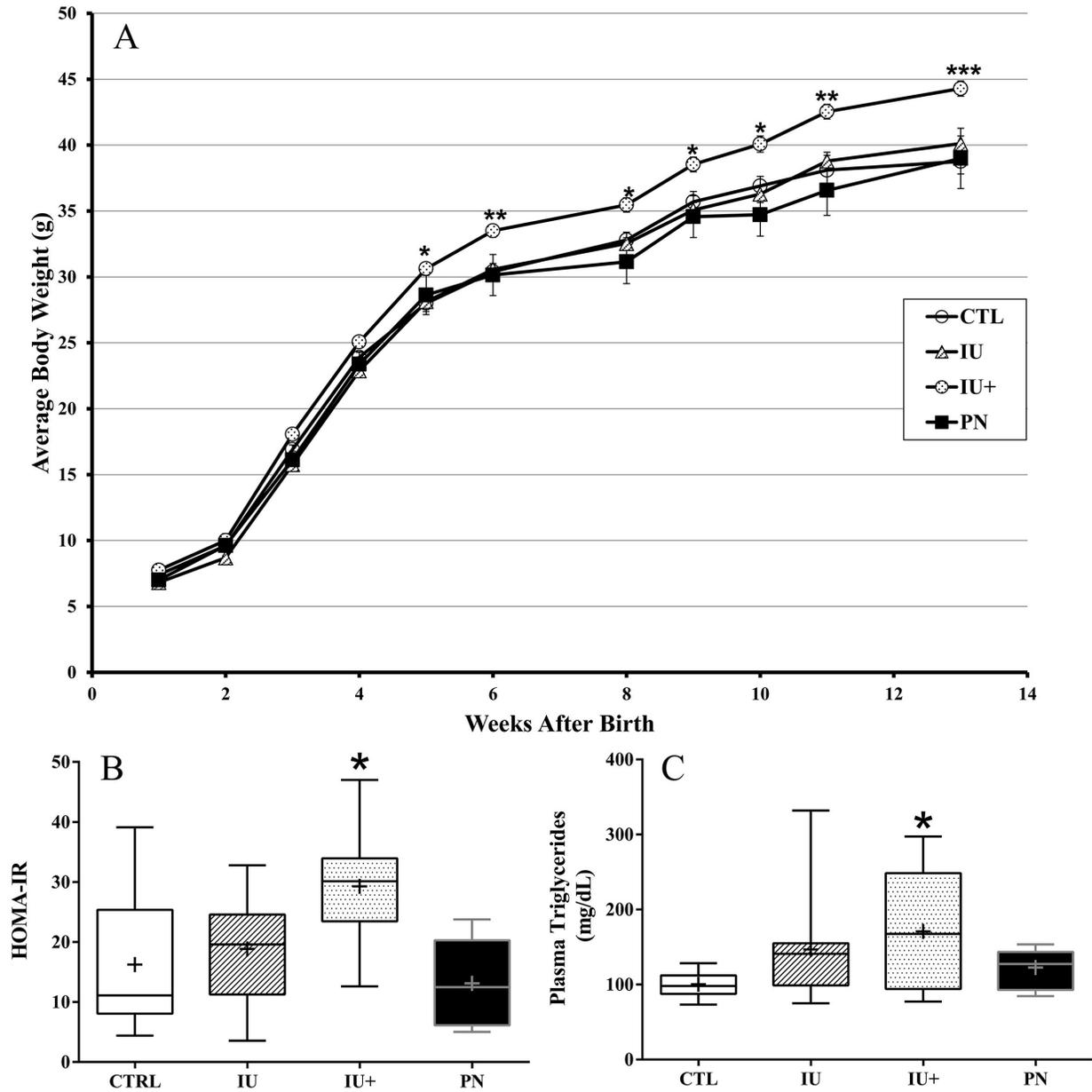


Figure 4

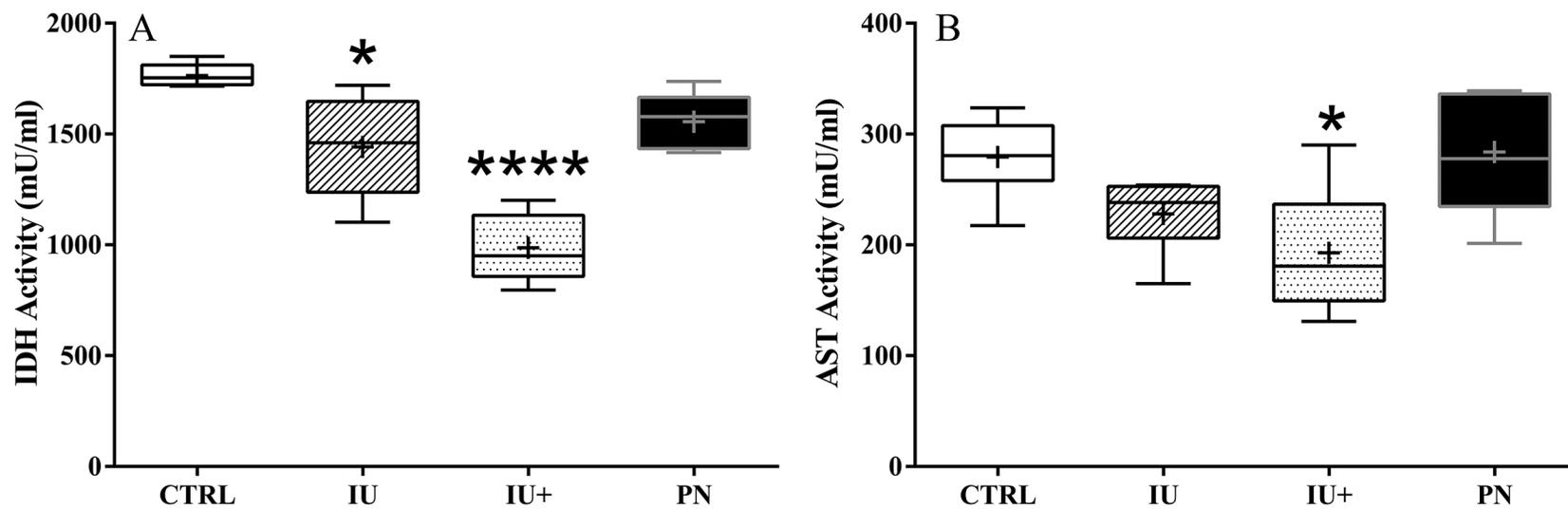


Figure 5

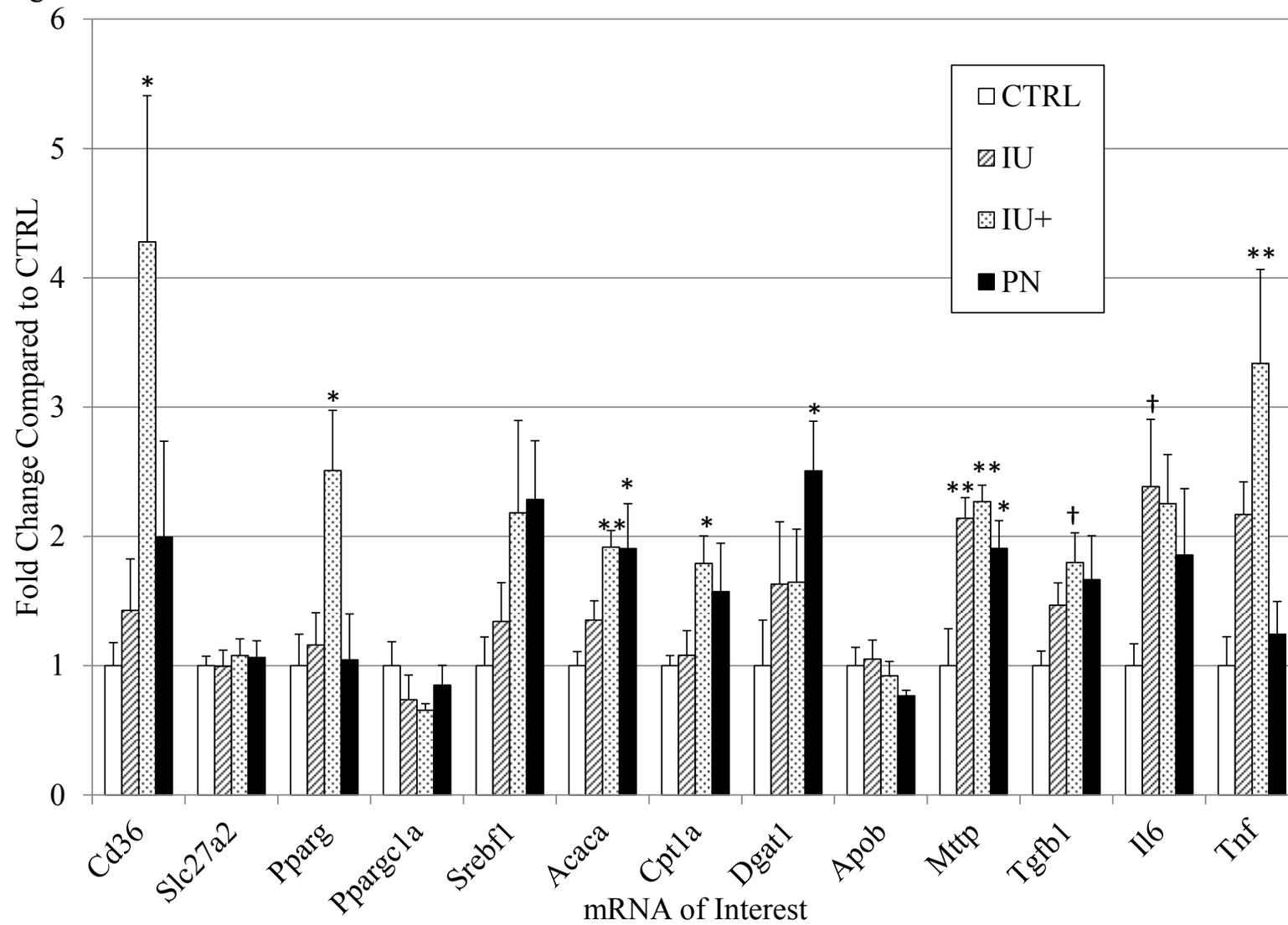


Figure 6

