Fetal exposure to arsenic results in hyperglycemia, hypercholesterolemia, and nonalcoholic fatty liver disease in adult mice

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Abstract

Background: Exposure to arsenic is a major concern in the United States and worldwide, since this metalloid has been associated with a number of ailments, including cardiovascular and metabolic diseases. Environmental exposures to toxicants throughout fetal development have been shown to play a critical role as triggers of adult disease.

Methods: This study aimed to evaluate the contribution of fetal arsenic exposure to the onset of metabolic syndrome. Swiss Webster mice were exposed to either 100 ppb sodium arsenite or sodium chloride via the dam’s drinking water from embryonic day 6 until birth. Weight and metabolic end-points were evaluated throughout the 36 week study. Retroorbital bleeds and blood plasma analyses were done to evaluate glucose, lipids, triglycerides and liver enzymes. Livers were evaluated histologically to assess extent and progression of nonalcoholic fatty liver disease. Cardiovascular outcomes such as blood pressure and ventricular hypertrophy were evaluated using non-invasive tail-cuff method and echocardiography respectively.

Results: Blood plasma analysis demonstrated that in-utero (IU) arsenic-exposed mice exhibited a significant increase in plasma glucose levels between weaning age and 4 months of age, which remained elevated after 8 months. Similarly, IU arsenic-exposed mice showed a consistent elevation in LDL and total cholesterol at weaning age, 4 months and 8 months of age. Mouse weight was not statistically different between groups, and no significant cardiovascular changes were seen. Further histological analysis of liver samples demonstrated the development of nonalcoholic fatty liver disease in IU arsenic-exposed mice, as evidenced by major morphological changes and an increase in steatosis concomitant with hepatocellular ballooning.

Conclusions: Taken together, the results found in this study suggest that IU arsenic exposure is a possible contributor to metabolic syndrome onset in mice, having important implications in the evaluation of fetal exposures on the development of adult disease.

Keywords: Arsenic, metabolic, fetal, hypercholesterolemia, hyperglycemia, NAFLD

Introduction

Exposure to environmental arsenic is a major concern in the United States and worldwide. It is estimated that hundreds of millions of people are exposed to arsenic through drinking water on a daily basis [1]. The highest concentrations seen in drinking water are found in the endemic Blackfoot disease regions of Taiwan where mean concentrations of arsenic in water are around 700 µg/L. Affected countries include Bangladesh, with over 30 million people exposed to concentrations between 0.1 µg/L and 864 µg/L, India, with 40 million people exposed to concentrations exceeding 1000 µg/L, as well as China (1.5 million people) and the United States (2.5 million people) exposed to concentrations between 1 µg/L and 100 µg/L of arsenic in the drinking water [2-5]. Importantly, exposure to arsenic has been associated with an array of diseases ranging from multiple forms of cancer, to developmental and reproductive effects, as well as cardiovascular and metabolic disorders [6-9].

Early life exposures to environmental toxicants such as arsenic have been shown to be strong influential factors in triggering the etiology of certain cancers [10], yet such exposures have not been as clearly established as contributors to cardiovascular disease or metabolic disorders. Several studies indicate that in utero (IU) and early life exposure to environmental toxicants play a critical role in increasing susceptibility to chronic ailments including metabolic dysregulation and cardiovascular disease [10-13]. A study performed by Yuan et al., in the region II of Chile provided key evidence supporting the idea that fetal exposure to arsenic is a strong contributor to increased cardiovascular mortality. Importantly, this region of Chile provided a unique opportunity to evaluate long-term health effects of early...
exposures to arsenic, since the period of high exposure was well defined by a sharp reduction of arsenic in municipal water. The study by Yuan et al., evaluated cardiovascular mortality ratios between 1950 and 2000, and demonstrated that young adults aged 30-49 who were born during the high exposure period with exposure IU and throughout early childhood, had the highest myocardial infarction mortality rates. These findings suggest that early exposure to arsenic promotes the development of cardiovascular disease throughout adulthood [10]. Similarly, multiple studies have shown that stress during pregnancy (such as those from exposures to environmental contaminants), and low birth weight during infancy are associated with an increased risk of coronary heart disease, hypertension, type 2 diabetes, and the development of metabolic syndrome in adulthood [14-18].

Additionally, Wang et al., show a linear relationship between arsenic content in hair (as a marker of exposure) and increased plasma glucose and lipids, as well as elevated blood pressure, associating arsenic exposure to an increased prevalence of metabolic syndrome [19]. Similarly, a recent study performed in South Korea showed that hair samples from patients with metabolic syndrome had significantly elevated arsenic content when compared with patients not diagnosed with metabolic syndrome [20].

Although metabolic syndrome is often classified as a disease, it is rather best described as a cluster of risk factors that together contribute to decreased cardiovascular and metabolic health. Metabolic syndrome is defined by the presence of 3 or more of the following risk factors: 1) Elevated waist circumference (men >40”; women >35”); 2) Elevated triglycerides (>150 mg/dl); 3) Reduced high density lipoprotein (HDL)(<40 mg/dl); 4) Elevated blood pressure (>130/85 mmHg); 5) Elevated fasting glucose (>100 mg/dl) [21,22]. Furthermore, steatosis—a process of lipid accumulation within liver cells—often leads to the development of nonalcoholic fatty liver disease (NAFLD), a hepatic manifestation of metabolic syndrome [23]. While steatosis is not a risk factor considered in the diagnosis of metabolic syndrome, it is often an important influence in the progression of this disease, negatively impacting liver function.

Metabolic syndrome has become a major public health concern and is a major cause of morbidity and mortality worldwide. It is estimated that the prevalence of metabolic syndrome among the general population is between 17 and 25% [24]. Additional reports within the United States suggest an even higher percent for the American population, at around 34% [25]. Various studies indicate that the major contributors to metabolic syndrome are insulin resistance, obesity and genetic predisposition; however, little is known about the contribution of environmental factors (such as arsenic exposure). Upon evaluating systemic effects of arsenic, it has been noted that oxidative stress contributes to the progression of diabetic vascular complications. While several studies support the hypothesis that arsenic exposure increases risk of type 2 diabetes, a lack in consensus exists due to experimental design limitations, making it difficult to evaluate a causal link [26]. Nevertheless, increasing biochemical data tend to strengthen this link between arsenic exposure and metabolic dysregulation [27-29]. Clearly, metabolic dysregulation is a complex, multifactorial condition that requires the evaluation of how each factor may contribute, including the effects of early-life exposures to environmental contaminants and how they may predispose or contribute to disease onset.

Cardiovascular and metabolic diseases develop as a result of complex interactions between genetic predisposition and lifestyle, including interactions with the surrounding environment both during development and throughout life. Despite increased awareness of the benefits of a healthier lifestyle, greater emphasis on diet and substantial improvement in pharmaceutical treatments, cardiovascular pathologies remain the leading cause of death. This suggests there still remains a significant gap in our understanding of contributing factors underlying cardiovascular disease and metabolic syndrome. As a basis for this study, we hypothesized that arsenic exposure during fetal development contributes to the onset and progression of metabolic disorders known to be risk factors for cardiovascular health. In order to assess this, we aimed to understand the metabolic outcomes resulting from fetal exposure to arsenic by evaluating biochemical, as well as physiological and histological markers of disease.

Methods

Animals and treatments

Swiss Webster pregnant mice were purchased from Harlan (Harlan Laboratories Inc, WI, USA). Treatment groups were initiated at embryonic day 6 (E6) by exposing the dams to either 100 parts per billion (ppb) sodium arsenite (NaAsO 2, Sigma, St. Louis, MO, USA) or 100 ppb sodium chloride (NaCl, VWR, Aurora, CO, USA) through drinking water, and were maintained on their respective treatments until birth. Mice were housed in sterile microisolator cages and provided diet (2019 Teklad Global 19% Protein Extruded Rodent Diet, Harlan Laboratories Inc, WI, USA) and water ad libitum. Offspring from arsenic exposed dams (n=2) consisted of 3 females and 7 males (n=10), whereas offspring from control dams (n=2) consisted of 8 females and 2 males (n=10). At weaning age (day 21), mice were separated by treatment and gender.

Systolic and diastolic blood pressures were measured biweekly using a volume pressure tail cuff transducer system coupled to computerized data acquisition software as previously described [9]. Mouse weights were recorded prior to acclimation to blood pressure analysis. Transthoracic echocardiography was performed on anesthetized animals at 2 and 6 months of age to assess left ventricular hypertrophy as described [9].

Blood samples were collected at weaning age, 4 months, and 8 months of age by retro-orbital puncture under a running laminar flow hood and sterile conditions. Approximately 250-300 µL of blood were collected from non-fasted mice between 12:00 PM and 1:00 PM. Autoclaved capillary tubes were used...
All plasma biochemical analyses were performed using the 4-chlorophenol. This oxidation product leads to a change in color directly proportional to the amount of triglycerides in the sample. Liver enzymes—Enzymatic activity based assays were used to colorimetrically measure liver enzyme concentrations in plasma samples. Alanine Transaminase (ALT) levels were calculated by reacting the sample with L-Alanine + 2-oxoglutarate, leading to the production of pyruvate, and reacted with lactate dehydrogenase in excess of NADH. This reaction leads to the production of NAD⁺ and a decrease in NADH, which can be measured at 340 nm, and is directly proportional to the amount of ALT. Aspartate Transaminase (AST) is measured in a similar fashion to ALT, by reacting L-aspartate + 2-oxoglutarate with the plasma sample, and then using malate dehydrogenase in the excess of NADH, to produce NAD⁺ and measure the decrease in absorbance of NADH. Alkaline Phosphatase (ALP) is measured by reacting p-nitrophenyl phosphate with the plasma sample to produce phosphate+p-nitrophenol, which is measured at an absorbance of 409 nm.

**Histology**

All organs were harvested and rinsed in phosphate buffered solution (PBS) (Mediatech, Herndon, VA, USA). Liver sections used for Hematoxylin & Eosin (H&E) and Masson’s trichrome stains were rinsed in PBS, and fixed in 4% paraformaldehyde (Fisher, Fair Lawn, NJ, USA) at 4°C overnight. Fixed tissue was embedded in Paraplast 56°C (McCormick Scientific, St. Louis, MO, USA), and sectioned in the transverse plane into 10 μm sections using a microtome (HM 325 Microtome, Thermo Scientific, Waltham, MA, USA). Additional tissue samples were embedded in Tissue-Tek OCT compound and snap frozen in liquid nitrogen for cryo-sectioning and Oil Red-O stain for lipid content analysis. Histology was documented using a DFC320 camera linked to a DM 2500 microscope (Leica Microsystems). The histological features of steatosis, fibrosis, inflammation and hepatocellular ballooning were semi-quantitatively evaluated in H&E and Masson’s trichrome stains through the (NAFLD) activity scoring (NAS) system as described [30]. Four liver sections were analyzed per mouse liver.

**Statistical analyses**

Mouse weights were averaged by gender and treatment groups, and analyzed using repeated measures analysis of variance (RM-ANOVA). Terminal liver enzymes (ALT, AST, and ALP) were averaged by treatment group and statistical significance was determined by the Student’s T-test. Blood plasma biochemistries were analyzed using a linear mixed effects model. This modeling procedure extends the usual linear model by including, in addition to fixed effects, random effects that account for correlations among observations in the data. In this case, mice were measured repeatedly over time and we attempted to capture the covariance structure of these dependent observations by modeling the within-mouse correlations. Specifically, we modeled the natural log concentrations of the blood biochemistry analytes with fixed effects including gender, treatment group, and time, as well as the interactions between them. The random effects included a unique intercept and time-associated slope for
Results

Body weight changes

Given the associations between cardiovascular, metabolic disorders and obesity, we evaluated growth changes during the 36 week period after birth. Female pups exposed to arsenic showed a statistically significant decrease in weight at two weeks of age, when compared to control females (p<0.05) (Figure 1A). While this difference in weight was apparent at 2 weeks of age, female mice recovered quickly, and by 4 weeks of age, there was no difference between IU arsenic-exposed and control mice (Figures 1A and 1B).

Blood plasma biochemistry

Epidemiological reports show that populations exposed to arsenic through drinking water have an increased prevalence of type 2 diabetes mellitus and cardiovascular disease [26,31-33]. Our study demonstrates that blood glucose levels did not significantly change over time in control mice (Figure 2A). In contrast, IU arsenic-exposed mice had a significant increase over time in blood glucose (46% increase) (p<0.01), as evidenced by a 39% increase between weaning age and 4 months (p=0.05), and an additional 7% increase between 4 months and 8 months of age (p=0.9). Further analysis of glucose levels in individual mice showed that control mice retained normoglycemic levels throughout the study (Figure 2B). The IU arsenic-exposed mice became hyperglycemic over time, and showed greater interindividual variability, as well as variability across the treatment group when compared to controls, strongly suggesting dysregulation of glucose homeostasis (Figure 2C).

Given our previous findings on arsenic-related cardiovascular pathologies [9], we evaluated cardiovascular outcomes as well as blood lipid profiles of these mice. While no significant
cardiac remodeling, or changes in systolic or diastolic blood pressures were observed between treatment groups (data not shown), a striking increase in circulating cholesterol was detected. The IU arsenic-exposed group was 26%, 44% and 42% higher than the control group at weaning age (p=0.08), 4 months (p=0.003) and 8 months (p=0.002) respectively. Further analysis of variation within groups demonstrated that neither control nor IU arsenic-exposed mice exhibited changes over time (Figure 3A). Similar to total cholesterol values, LDL cholesterol values (Figure 3B) were consistently elevated in the IU arsenic-exposed animals at 4 months (p=0.0001) and 8 months (p=0.034), when compared to controls, without a significant change over time. HDL cholesterol values for the IU arsenic-exposed mice were significantly elevated only at the 4 months of age (p=0.03); however, a trend towards elevated HDL was apparent at weaning age and 8 months of age when compared to controls (Figure 3C). Evaluation of triglyceride levels revealed no significant change between IU arsenic-exposed and control mice (Figure 3D). While, a significant decrease was observed in the IU arsenic-exposed group between weaning age and 4 months of age (p=0.02), the change was not present by 8 months of age. 

Liver histology
The detection of increased circulating lipids in the plasma of IU arsenic-exposed mice prompted investigation of livers for pathology. Gross examination of livers from control mice revealed no obvious signs of steatosis, whereas in 70% of IU arsenic-exposed mouse livers, consistent discoloration indicative of steatosis was observed (4 slides per liver). Histological examination of hematoxylin and eosin staining revealed normal liver architecture in control mice (Figure 4A). In contrast, the livers of IU arsenic-exposed mice exhibited prominent micro and macrovesicular steatosis. Macrovesicular steatosis and hepatocellular ballooning are evident (black arrows) as well as contiguous microvesicular steatosis (white arrows) in the IU arsenic-exposed group (Figure 4C). Livers of control mice showed minor signs of lipid accumulation as detected by Oil Red-O staining (Figure 4B), whereas the livers of IU arsenic-exposed mice had substantially larger lipid droplet accumulation (Figure 4D, gray arrows). Masson’s trichrome staining was performed to assess fibrotic changes; however, no overt alterations were observed between livers of control (Figure 5A) and IU arsenic-exposed mice (Figure 5B). Consistent with these observations, liver damage evaluated by analysis of terminal circulating plasma ALT, AST, and ALP, demonstrated no significant differences between control and IU arsenic-exposed mice (Figure 5C). Additionally, the NAFLD activity score (NAS) was used as a semiquantitative scoring system useful for assessing histological features of NAFLD progression. Control animals showed little to no signs
of steatosis and no indications of hepatocellular damage. In contrast, livers from IU arsenic-exposed mice showed contiguous microvesicular steatosis (+1) (white arrows), as well as hepatocellular ballooning (+1) (black arrows). Fibrosis, megamitochondria or inflammation were not observed (+0) in IU arsenic-exposed animals, resulting in an overall NAFLD activity score of +2. This suggests a NAFLD-(not NASH (nonalcoholic steatohepatitis)) state, as defined by a total NAS value of <3 [30].

Discussion
Chronic arsenic exposure has been well studied and evaluated in the context of adult disease; however, little is known about the contribution of fetal arsenic exposure to disease onset and progression. Several studies established a strong association between chronic arsenic exposure and an increased prevalence of hypertension [34,35], atherosclerosis [36,37], and ischemic heart disease-related mortalities [38,39]. Importantly, these epidemiological studies have shown that the relationship between cardiovascular disease and arsenic exposure is not only dependent on dose, but also on duration of exposure. Seldom have studies evaluated the contribution of developmental exposures. However, Smith’s group [10] provided strong evidence for the association between arsenic exposure through drinking water, and a sustained increased risk in circulatory disease-related mortalities, years after exposure. More importantly, the studies by Smith’s group provided some of the first evidence in humans of the detrimental health effects from IU arsenic exposure resulting in an increased incidence of myocardial infarction in adults [10]. Similarly, arsenic exposure has been linked to NAFLD, obesity and diabetes, even though the exact the molecular mechanisms are not fully understood [40]. Although these associations have been shown before, to our knowledge, no reports of fetal arsenic exposure exist showing an association to NAFLD.

Obesity is a key component of metabolic syndrome, and it
is also an important risk factor for cardiovascular disease; as such, the weight of each mouse was monitored throughout the study. An interesting observation was that the female mice exposed to arsenic IU were significantly underweight during the first two weeks after birth, when compared to their control counterparts. Similarly, Kozul-Horvath et al., showed that female mice that were exposed to 10 ppb arsenic IU, had a delay in weight gain at postnatal day 21 [8]. Our IU arsenic-exposed mice showed a full recovery in weight by 4 weeks of age, and no significant changes were observed thereafter. Although a trend towards higher body weights was observed for both males and females, there was no statistical significance when compared to their control counterparts.

The array of biochemical and clinical markers of metabolic syndrome and cardiovascular disease observed in this study are of major significance, considering that exposures to 100 ppb doses are quite prevalent throughout the world [2-5]. The progression of hyperglycemia between weaning age and 4 months of age is similar to what would be expected from the development of insulin resistance in type 2 diabetes mellitus. The most frequent cause of hyperglycemia in diabetes mellitus results from a deficiency in insulin secretion or action. However, insulin levels were not measured successfully in our plasma samples. One of the pitfalls of insulin detection in serum is that the occurrence of hemolysis, and sample freeze-thawing results in substantial degradation and matrix interference during detection [41,42]. All of these limitations, compounded by the limited sensitivity of insulin immunooassays, make insulin detection methods particularly challenging. It is also possible that the observed hyperglycemia is linked to the development of NAFLD, and therefore, additional studies are needed to investigate the effects of fetal arsenic exposure on insulin levels. Nevertheless, the observation that glucose levels increased over time in the IU arsenic-exposed mice is consistent with the epidemiological studies associating type 2 diabetes mellitus with arsenic exposure [29,31], as well as with molecular mechanism and animal studies demonstrating that arsenic may impair insulin response and sensitivity [27,28,43,44].

Elevated cholesterol is an important risk factor associated with metabolic syndrome that contributes to its association with reduced cardiovascular health. Specifically, elevation of LDL cholesterol is a major component causing and influencing atherogenesis and coronary heart disease, and it is the single most important clinical predictor of coronary artery disease [45-48].

A limitation of using a mouse model in this case, is that it provides limited insights to the significance of the observed increase in cholesterol with respect to cardiovascular health in humans, since non-genetically-predisposed mice have very low LDL cholesterol values, and turnover rates are different when compared to humans. Typically, mouse LDL hepatic clearance rates reach 500 ml/day/kg, which is about 40-fold greater than human hepatic LDL clearances [49]. Consequently, the steady-state concentration of LDL cholesterol in wild-type mice is approximately 7 mg/dl, whereas in humans this value usually exceeds 100 mg/dl [50]. Nevertheless, this study demonstrates that a substantial increase in circulating LDL cholesterol was present in mice exposed to arsenic during fetal development, and the ramifications of this observation are likely to have an impact in the context of cardiovascular health, and should be evaluated closely in human populations exposed to arsenic in utero.

Similar to the increase in LDL cholesterol, IU arsenic-exposed mice had elevated HDL values at 4 months. While the underlying mechanisms behind this observation remain unclear, we postulate that this is a reflection of the impaired cholesterol metabolism, and not a beneficial effect of arsenic exposure.

Circulating triglycerides were analyzed in control and IU arsenic-exposed mice. Similar to Srivastava’s observations in the ApoE−/− mouse model [37], a significant decrease in triglycerides was observed in the IU arsenic-exposed group between weaning age and 4 months of age. Interestingly this decrease was not detected by the 8 month time point. Whether these observations are a result of compensatory or adaptation mechanisms remains unclear. Nevertheless, it is believed that LDL cholesterol, rather than triglycerides, is the main contributor to the progression of cardiovascular diseases such as atherosclerosis. Increased levels of non-fasting triglycerides often indicate the presence of an atherogenic state; however, all human cells are able to degrade triglycerides, but not cholesterol. It is, therefore, the LDL cholesterol content that can cause atherosclerosis upon entrance into the arterial intima [51,52]. Additionally, detection of triglycerides and lipid accumulation in the livers of IU arsenic-exposed mice provides strong evidence that early arsenic exposure promotes the onset and progression of NAFLD. In humans, increased risk of cardiovascular disease is associated with NAFLD, as it is often a co-morbidity that develops in metabolic syndrome [53,54]. Furthermore, NAFLD is increasingly recognized as the hepatic manifestation of insulin resistance and metabolic syndrome [55]. Importantly, NAFLD is estimated to be prevalent in 20-30% of the general population in western countries [56], so understanding the underlying causes for the onset and progression of this disease is essential. In this regard, our results are consistent with previous studies showing that arsenic exposure leads to fatty livers in pregnant female mice [8]. Thus, arsenic may be an important toxicant with particular sensitivity to the developing fetus, predisposing mice to NAFLD.

To evaluate plasma biochemistry, mice were in the non-fasted state prior to blood collection. There were two reasons behind this. First, we believe that atherogenesis and cardiovascular disease progression are largely a postprandial phenomenon. Humans spend a larger portion of their lives in the postprandial state, while paradoxically, the vast majority of studies assessing cardiovascular disease risk factors are conducted in a fasted state. Importantly, it has been demonstrated that fasted triglyceride values do not represent an independent risk factor
for cardiovascular disease, whereas studies in the postprandial state show that triglycerides can be used as a predictor of coronary heart disease [57], myocardial infarction, and ischemic heart disease-related deaths [58-60]. Similarly, in type 2 diabetes mellitus patients, non-fasting blood glucose level has been shown to be a better predictor of cardiovascular events than fasting blood levels [61,62]. Secondly, as mentioned previously, the mice in this study were not genetically predisposed to develop diabetes or atherosclerosis, so we believed that this represented a possibility for false negative results, given enough time in the fasted state.

When designing studies of IU exposure, controlling for variables like gender ratio between treatment groups becomes challenging, since treatment is determined prior to identification of gender of the offspring. By using the mixed effects model, we are able to accommodate the unbalanced data and attempt to control for the gender effect. However, given this gender imbalance between the control and IU arsenic-exposed groups, it becomes a challenging task to tease apart what extent treatment and gender contribute independently to the observed effects. While our model did reveal a statistically significant treatment effect (independent of gender effect), confirmation of these findings with larger numbers of animals would need to be conducted to better assess the contribution of gender, independent of other variables, to arsenic triggered diseases. Furthermore, it is possible that gender specific pathophysiological differences exist in the onset of metabolic syndrome [63].

Conclusions
In summary, we demonstrate that fetal exposure to arsenic results in the development of multiple risk factors for the development of metabolic syndrome, which are also significant contributors to cardiovascular-related mortalities. The presence of several components of metabolic syndrome in mice suggests that fetal exposures to arsenic may contribute to dysregulation of regulatory pathways important for glucose and lipid homeostasis. Mechanisms of arsenic-mediated metabolic disruption go beyond the scope of this manuscript, and are discussed in great reviews [26,64-66]; however, preliminary findings from our lab indicate that arsenic may be affecting the function of key enzymes involved in energy metabolism and the tricarboxylic acid (TCA) cycle. Given the pivotal role that the liver plays in glucose and lipid homeostasis, it is likely that disruption of a central bioenergetics pathway such as the TCA cycle could contribute to the effects observed in this study.

The findings presented here are novel and suggest an association between IU arsenic exposure and metabolic syndrome that has not been evaluated before. Collectively, these results suggest that developmental exposure to arsenic is a previously unrecognized contributor to the onset of metabolic syndrome, and likely increases the risk of developing cardiovascular disease; however additional investigation is necessary to understand the implications these findings may have in human health.

List of abbreviations
ALP: Alkaline Phosphatase
ALT: Alanine Transaminase
AST: Aspartate Transaminase
HDL: High Density Lipoprotein
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
NAFLD: Nonalcoholic Fatty Liver Disease
NAS: NAFLD Activity Scoring
PBS: Phosphate Buffered Solution
PPb: parts per billion
RM-ANOVA: Repeated Measures Analysis of Variance

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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