RENAL HEALTH EFFECTS IN TRICHLOROETHYLENE AND ARSENIC CO-EXPOSED MICE

A Dissertation

by

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ABSTRACT

Development of a rodent model for renal cancer and disease development due to toxicant exposure is complicated by differences in renal handling of toxicants between rodents and humans as well as the tendency of rodents to develop significant background spontaneous renal disease that may mimic pre-neoplastic disease or mask more subtle lesions. Classical toxicological studies often focus on one toxicant in a genetically homogeneous population, despite attempting to model human exposure situations involving genetically heterogeneous populations and exposure to mixtures of toxicants. Given these challenges, it has become clear that toxicological studies must address the effects of genetic variability and the range of sensitivity to toxicity due to this inherent variability. Experimental paradigms that assess and control for as many of the intrinsic and extrinsic factors influencing renal response to toxicant exposure are also needed. In an effort to address these limitations, a mouse model was devised that included genetic heterogeneity, mixtures of toxicants at environmentally relevant concentrations, and diet that reflects a typical western diet to better reflect the exposure conditions of human populations.

Despite development of a mouse model that more accurately reflects human environmental toxicant exposure conditions, no primary renal cell tumors were observed in the study. Differences in renal health between exposed and unexposed populations were observed as well as increased evidence of renal disease in male mice compared to females across the entire study population. In the current study TCE exposure did not

cause renal cell tumors nor did it increase renal disease when combined with arsenic exposure. Evidence of reduced or equal renal damage from co-exposure was observed in some cases, and we speculate that this is due to a threshold effect of damage from a first toxicant limiting the ability of a second toxicant to cause damage. Additional studies of combined toxicant exposure are needed.

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NOMENCLATURE

BUN	Blood Urea Nitrogen
CDC	Centers for Disease Control and Prevention
CKD	Chronic Kidney Disease
H&E	Hematoxylin and Eosin
iAs	Inorganic Arsenic
MDR	Multi-Drug Resistance
NGAL	Neutrophil Gelatinase-Associated Lipocalin
RCC	Renal Cell Carcinoma
SNP	Single Nucleotide Polymorphism
TCE	Trichloroethylene
QTL	Quantitative Trait Locus

TABLE OF CONTENTS

Page

ABSTRACT ii

ACKNOWLEDGEMENTS iv

CONTRIBUTORS AND FUNDING SOURCES v

NOMENCLATURE vi

TABLE OF CONTENTS vii

LIST OF FIGURES ix

LIST OF TABLES x

1. INTRODUCTION 1

1.1. Significance 1
1.2. Trichloroethylene 3
1.3. Toxicant Mixtures in Environmental Exposures 9
1.4. Arsenic 10
1.5. Genetic Heterogeneity in Toxicological Testing 15

2. RENAL TUBULAR DAMAGE BUT NOT TUMORIGENESIS RESULTS FROM LONG-TERM COMBINATORIAL EXPOSURE TO TRICHLOROETHYLENE AND INORGANIC ARSENIC IN GENETICALLY HETEROGENEOUS MICE DEFICIENT FOR MULTI-DRUG RESISTANCE 17

2.1. Overview 17
2.2. Introduction 18
2.3. Materials and Methods 22
2.3.1. Animals 22
2.3.2. Toxicant Exposure 26
2.3.3. Sample Collection and Histopathologic Examination 27
2.3.4. Statistical Analysis 28
2.4. Results 28
2.4.1. Survival is Not Affected by Exposure Group 28
2.4.2. Exposure is Associated with Renal Tubular Disease 30
2.4.3. Exposure and Sex Influence of Non-Renal Neoplasia 36

2.5. Discussion 36

3. TOXICANT EXPOSED AND UNEXPOSED MALE MICE HAVE HIGHER RENAL DAMAGE BUT RENAL DAMAGE IS NOT INCREASED BY COMBINATION EXPOSURE TO TRICHLOROETHYLENE AND INORGANIC ARSENIC 41

3.1. Overview 41
3.2. Introduction 42
3.3. Materials and Methods 46
3.3.1. Animals and Toxicant Exposure 46
3.3.2. Sample Collection and Analysis 47
3.4. Results 51
3.4.1. Male Mice Have Greater Renal Damage 51
3.4.2. Inorganic arsenic does not increase histologic evidence of tubular damage in combination with low TCE 53
3.4.3. BUN Levels – Correlation to Histology Parameters and Levels Among Groups 57
3.4.4. Urinary NGAL 60
3.4.5. Urine Protein/Creatinine Ratio 63
3.5. Discussion 65

4. CONCLUSIONS 72

4.1. Modeling of Biological Response to Toxicant Exposure 72
4.2. Future Work 76

REFERENCES 82

LIST OF FIGURES

Page

Figure 2.1 Breeding Plan. 24
Figure 2.2 Examples of Renal Pathology. 31
Figure 2.3 Tubular Disease Score by Exposure Group. 34
Figure 2.4 Tubular Disease Score by Toxicant Exposure Status. 35
Figure 3.1 Renal Parameters for Male Versus Female Mice. 53
Figure 3.2 Interaction Between iAs and TCE Level. 55
Figure 3.3 Histologic Lesions in Toxicant-Exposed vs. Non-Exposed Mice. 57
Figure 3.4 BUN Level by Toxicant Exposure Group. 60
Figure 3.5 Urinary NGAL by Toxicant Exposure Group. 62
Figure 3.6 Urine Protein Creatinine Ratio by Toxicant Exposure Group. 64
Figure 3.7 UPC Ratio in Male vs. Female Mice 65
Figure 4.1 BUN Extreme Responders 78
Figure 4.2 Tubular Disease Score Extreme Responders 79
Figure 4.3 Urinary NGAL Normalized to Creatinine Extreme Responders. 80
Figure 4.4 Overlap in Extreme Responders for Tubular Disease, BUN, and NGAL. 81

LIST OF TABLES

Page

Table 2.1 Toxicant Dose Groups....26

Table 2.2 Deaths per Group During Study Period....29

Table 2.3 Male and Female Deaths per Group....30

Table 2.4 Tubular Disease Scoring Criteria....32

Table 2.5 Renal Neoplasia....36

Table 3.1 Correlation of Biomarkers with Gross and Histologic Renal Disease....58

1. INTRODUCTION

1.1. Significance

Chronic exposure to environmental toxicants has been linked to a number of health effects including various cancers and kidney disease (Boffetta, 2004; Kataria *et al.*, 2015; Rusyn *et al.*, 2014; Soderland *et al.*, 2010; Van Vleet *et al.*, 2003). The kidney is frequently a target organ for toxicants due to its role in filtration of the blood and metabolism of exogenous substances (Van Vleet, et al., 2003). Because of this role, it is reasonable to expect that exposure to many environmental toxicants would result in kidney damage, disease, and possibly cancer.

Cancers of the kidney and renal pelvis account for 3.8% of all new cancer cases in the United States and an annual incidence of 15.6 per 100,000 people (National Cancer Institute). Tumors of renal tubular cells, renal cell carcinomas, comprise nearly 90% of renal malignancies. Malignant tumors of the renal pelvis, which are mainly transitional cell carcinomas, account for approximately 10% of renal tumors. Nephroblastoma, which is a malignancy of the kidney typically observed in children, accounts for only about 1% of renal malignancies.

The incidence trend for renal cancer has continued to increase from the 1970s through the present (National Cancer Institute, 2017), although the cause for this has not been determined, and the trend shows evidence of leveling off in recent years. In some areas of the country, often overlapping with National Priority List (Superfund) sites, renal cancer incidence is higher than the national average. One such location is Onslow

County, North Carolina, home to a Superfund Site (Camp Lejeune) contaminated with trichloroethylene (TCE) and inorganic arsenic (iAs). According to the State Cancer Profiles from the Center for Disease Control and Prevention (CDC) and NCI, Onslow County had an annual renal cancer incidence of 21.3 per 100,000 from 2008-2012, well above the national average.

While kidney cancer is the 8th most commonly diagnosed cancer in the United States each year (National Cancer Institute), an even larger cause of morbidity and mortality is chronic kidney disease (CKD). Many different etiologies can be responsible for the initial kidney injury, including environmental toxicants (Kataria, et al., 2015; Yang *et al.*, 2010). Kidney disease is progressive, and eventually, regardless of the instigating problem, the result is the same: CKD and eventual development of end stage kidney disease resulting in the need for hemodialysis and transplant. Consequently, kidney diseases are the 9th leading cause of death in the United States (Murphy SL *et al.*, 2017).

CKD is characterized by permanent damage and subsequent loss of functional nephrons with eventual decline in glomerular filtration rate (a measure of kidney function). The CDC estimates that 30 million people, or 15% of the United States population has CKD (Centers for Disease Control and Prevention, 2017). Because remaining functional nephrons can compensate until about 75% of nephrons are lost, almost half of those with severe kidney disease are not aware that they have the disease. Due to the lack of clinical signs associated with earlier stages of kidney disease, and the lack of sensitive and specific diagnostic screening tests, CKD is often not diagnosed

until later stages, when little can be done to treat the disease beyond dialysis and, eventually, renal transplant.

Renal cancers and CKD share some common risk factors including obesity, smoking, and hypertension. Exposure to some environmental toxicants, including TCE and arsenic, has been established as a risk factor for the development of kidney cancer (National Center for Environmental Health, 2016). Recently, studies have linked chronic kidney disease with environmental toxicants, especially chronic cumulative exposure. However, CKD is multifactorial, and genetic susceptibility and other health conditions likely play a large role in the development of this disease (Kataria, et al., 2015; Soderland, et al., 2010).

Because people are often exposed to mixtures of environmental toxicants, it is imperative that the effects of combination exposures to environmental toxicants on kidney disease and cancer are studied in a model that closely mirrors the conditions that humans are likely to encounter. Two common environmental toxicants, trichloroethylene (TCE) and inorganic arsenic (iAs), are the focus of this study.

1.2. Trichloroethylene

Trichloroethylene (TCE) is a chlorinated solvent that was used heavily as an industrial degreaser in the United States starting in the 1900s, in batch cleaning in the dry cleaning industry, and as part of household aerosol products including paint strippers and adhesives, but whose use in this country has been drastically reduced since the

1970s (Bakke *et al.*, 2007). TCE is still in use, although in much reduced volume, as an intermediate in the production of hydrofluorocarbon refrigerant and as a metal degreaser.

Trichloroethylene is not naturally encountered in the environment, but because of its historical wide use and improper disposal, TCE has been found at the majority of the current and proposed Superfund sites (Scott *et al.*, 2000), and it is the most commonly reported organic groundwater contaminant (National Research Council (U.S.) Committee on Human Health Risks of Trichloroethylene., 2006). According to the Agency for Toxic Substances and Disease Registry (ATSDR), 4.5-18% of US drinking water sources tested by the EPA contain detectable levels of TCE (Agency for Toxic Substances and Disease Registry, 2014). According to the most recent data available, in 2009-2013 an estimated 53 million people lived within a 3-mile radius of a Superfund site (U.S. EPA Office of Solid Waste and Emergency Response Estimate, 2015). Proximity to a Superfund site does not necessarily mean an increased risk of health effects due to the difference in contaminants and containment. Even so, the number of people living near Superfund sites does highlight the potential for exposure to environmental toxicants, especially should containment or mitigation efforts be insufficient.

The majority of TCE used in the United States today evaporates into the air, and its half-life in air is approximately 7 days (Agency for Toxic Substances and Disease Registry, 2014). Unfortunately, TCE has high mobility in soil and through this route can contaminate groundwater before evaporation can disperse it. Once in underground water sources, TCE may persist because it is slow to degrade in the underground environment

(Agency for Toxic Substances and Disease Registry, 2014). TCE has also been detected in marine sediments as well as marine invertebrates and mammals, and some foods. In fact, butter and margarine are foods with the highest reported mean concentration of TCE at 73.6 ppb (Environmental Protection Agency, 2001).

A statistically-based national sampling program for TCE in the environment has not occurred and so there is no true estimate of the mean level of TCE in any environmental medium (Environmental Protection Agency, 2001). Some data regarding air levels are available, and generally levels of TCE in the air are low, especially in rural areas. Air levels of TCE are highest in the vicinity of industrial use.

As previously stated, TCE can be found in groundwater. One potential source for much of the groundwater contamination is leaching of TCE through the soil from landfills. Several studies have found that landfill leachate can contaminate nearby groundwater supplies (Agency for Toxic Substances and Disease Registry, 2014; Dewalle *et al.*, 1981; Schultz *et al.*, 1986), and the release of TCE into soil occurs in much larger volume than its release into water supplies. While it isn't known how often the level of TCE in groundwater reaches a level that would have toxic effects, wells located near areas of TCE use and disposal can be anticipated to have the highest risk of contamination (Environmental Protection Agency, 2001). As stated above, when tested by the EPA, between 4.5 and 18% of drinking water sources in the United States had detectable levels of TCE (Agency for Toxic Substances and Disease Registry, 2014). In 2005, EPA screening of the public water system detected TCE in 2,292 of 46,937 (4.9%)

of samples collected from groundwater. The maximum concentration detected was 159 ppb, although the median was only 1.1 ppb.

Workers in industries that utilize TCE, such as degreasing operations, have the highest exposure levels to TCE. This exposure is mostly through inhalation, and exposure may be as high as 100 ppm. The population at large is more usually exposed through contaminated water or food or by contact with consumer products that contain TCE (Agency for Toxic Substances and Disease Registry, 2014). TCE has been detected in human milk, meaning that breastfeeding babies may be exposed through that route as well, if the mother is exposed.

According to an EPA report on the health risks of TCE, the range of estimated exposure (in air) to TCE in the general adult population is 11-33 μg/day. In water the estimated exposure in the general population is 2-20 μg/day. For people who work with TCE, the exposure through air ranges from 2232-9489 μg/day. People may also be exposed to metabolites of TCE in the environment, such as trichloroacetic acid (TCA) and dichloroacetic acid (DCA), and these exposures may be much higher than the exposure to TCE itself, which may increase the toxic effects of TCE exposure (Environmental Protection Agency, 2001).

Exposure to TCE has been associated with a variety of cancers and disorders, including renal cancers and renal injury (Liu *et al.*, 2010; Wartenberg *et al.*, 2000), and it has been associated with increased mortality from kidney cancer (Alanee *et al.*, 2015). The National Cancer Institute (NCI) estimated that almost 64,000 new cases of kidney

and renal pelvis cancer were diagnosed 2017, accounting for approximately 3.8% of all new cancer cases in the United States (National Cancer Institute).

As previously mentioned, populations in some areas have higher incidences of renal cancer than in the general population, and these increased incidences are sometimes found to correspond to Superfund sites, as is the case with Camp Lejeune, mentioned previously. Camp Lejeune was home to a Superfund Site contaminated with TCE between the 1950s and 1985 and had an increased incidence of renal tumors compared to the national average from 2008-2011.

Two water supply wells on Camp Lejeune were contaminated; one from an off-base dry-cleaning operation and one from on-base sources (Bove *et al.*, 2014). While both of these contaminated water sources were shut down by 1985, many people stationed at this base were exposed to a mixture of toxicants including TCE and tetrachloroethylene, a related compound. The maximum detected level of TCE before the contaminated wells were shut down was 1400 μg/L, while the maximum contaminant level (MCL) set by the EPA as the upper limit for TCE in water supplies was 5 μg/L (Bove, et al., 2014).

A retrospective cohort study of those stationed at Camp Lejeune between 1979 and 1985 (during the contamination period) found that there was an elevated risk of mortality for those stationed at Camp Lejeune compared to the general population of the United States during this period for several causes of death, including cancers of the kidney and liver which are targets of TCE (Bove, et al., 2014). Follow up in this study only extended through 2008, when 97% of the remaining cohort was still under age 55,

leaving comprehensive analysis of the long-term risks from exposure on the base for future studies. One limitation of the study was that correlations for the various related toxicants were very similar, making it impossible in many cases to separate the effects of the individual contaminants. Even so, given the high levels of TCE and related toxicants, this study provides further evidence of the association between TCE exposure and kidney cancer.

In addition to its association with the development of RCC, TCE has been associated with a variety of other cancers and disorders, including renal injury, cancers of hematopoietic tissues, and nervous disorders (Environmental Protection Agency, 2001; Liu, et al., 2010; National Toxicology Program, 2014; Wartenberg, et al., 2000). Hydrocarbons such as TCE have been associated with the development of CKD (Radican *et al.*, 2006; Ravnskov, 2000; Wedeen, 1992). Indeed, Radican *et al.* found that there was a 2-fold greater risk of developing end-stage kidney disease with TCE exposure. Additional factors that mediate the severity of TCE toxicity and the development of renal cell carcinomas include gender, smoking tobacco, obesity, chronic renal failure, hypertension, and exposure to other environmental toxicants, such as inorganic arsenic (American Cancer Society, 2015; Lash *et al.*, 2001).

TCE is lipophilic and rapidly distributes through the body after exposure (Cristofori *et al.*, 2015). Its main metabolism is through the cytochrome P450 system (mostly CYP2E1) in the liver (Kim *et al.*, 2006; Lock *et al.*, 2006). Here, it is oxidized with ultimate production of the main TCE metabolites trichloroethanol (about 30%) and trichloroacetic acid (about 10%) (Lock, et al., 2006). An alternate pathway for TCE

metabolism occurs in the kidney. In the kidney glutathione (GSH) conjugation occurs, forming S-(1,2-dichlorovinyl)-L-cysteine (DCVC) followed by other reactive metabolites (Cristofori, et al., 2015; Lock, et al., 2006). This metabolic pathway is minor in humans, accounting for metabolism of approximately 0.01% of the TCE exposure dose (Cristofori, et al., 2015; Lock, et al., 2006).

Since mice produce far more oxidative metabolites when exposed to TCE than GSH conjugation derived metabolites (Cichocki *et al.*, 2016; Kim *et al.*, 2009; Luo *et al.*, 2018; Yoo *et al.*, 2015a; Yoo *et al.*, 2015b), it appears that GSH conjugation is the minor pathway for TCE metabolism in mice. While less information is available about the relative efficiency of each pathway of TCE metabolism in humans, it appears that, in general, humans are far less efficient at metabolizing TCE, leading to potentially longer exposure to this toxicant. This difference in efficiency may lead to a longer half-life for TCE and its metabolites in humans and might help explain the discrepancies in effects between mice and humans, despite similar metabolism of TCE (Kim, et al., 2009). Generally, it is believed that metabolites of the oxidation pathway are responsible for adverse health effects in the liver and that metabolites derived from GSH conjugation are responsible for kidney health effects (Bull, 2000; Lash *et al.*, 2000b).

1.3. Toxicant Mixtures in Environmental Exposures

There has recently been increased interest and study on the effects of chemical or toxicant mixtures on health outcomes (Kapraun *et al.*, 2017; Pollock *et al.*, 2017), particularly for toxicants like TCE whose effects on disease severity and outcome may

be mediated by co-exposures in the environment. The effects from mixtures of toxicants cannot be predicted based on simple additive assumptions (Berenbaum, 1989), therefore studying mixtures of toxicants is important to determine the actual health effects that can be expected due to these exposures.

Typically, toxicology studies use a single strain of rodent and one toxicant at a time to assess the effects of a potential toxicant. One of the main groups involved in toxicological testing utilizes the B6C3F1 mouse, a hybrid between the C57BL/6 and C3H inbred mouse strains, and the Harlan Sprague Dawley rat for carcinogenicity studies (King-Herbert *et al.*, 2010). For decades (until 2006), the Fisher 344 rat was used in these studies, but use of this strain was discontinued by the NTP due to high background incidence of some tumors. This study of toxicants in isolation and in genetically homogeneous populations does not model real-life toxicant exposures, as people are usually exposed to mixtures of substances and are definitely not genetically homogeneous. Inorganic arsenic (iAs) is a prime candidate for studying the interactions of multiple toxicants in a more controlled setting than is possible using human retrospective studies because it is commonly encountered. Because it is so common, arsenic is likely to be part of a mixture of toxicants to which a population might be exposed.

1.4. Arsenic

Arsenic is a metalloid element with four oxidation states, the two predominant states being trivalent arsenite (As III) and pentavalent arsenate (As V) (Iarc Working

Group, 2012; National Toxicology Program, 2014; WHO, 2001). As III is more readily absorbed and has been more associated with toxicity (Hindmarsh *et al.*, 1986), although metabolism of As IV results in production of As III as well, so both are toxic. Arsenic is often found as a compound rather than in pure elemental form. Inorganic arsenic is very bioavailable and considered to be of more concern for toxic effects than organic arsenic, since organic sources of arsenic are generally present at lower levels (Iarc Working Group, 2012).

Arsenic has been in use for hundreds of years in many different applications including, but not limited to, wood preservation, pesticides, mining, and pharmaceuticals (Iarc Working Group, 2012; Kimura *et al.*, 2005). Arsenic is a common element in the Earth's crust and can also be released into the environment through anthropogenic activities such as mining and burning of fossil fuels. In addition to deposition from these sources, large areas of land are contaminated with inorganic arsenic from its historical use as a pesticide on agricultural land.

Water is the primary transport medium for arsenic in the environment, and the level and type of arsenic contamination depends on several factors, including oxygenation of the water, type of water source, biological activity, and proximity to sources of arsenic (WHO, 2001). Levels of arsenic in water can vary from less than 10 µg /L (averaging 1-2 µg /L in groundwater) up to 5 mg/L near anthropogenic sources or 3 mg/L in areas with high volcanic rock content. Arsenic contamination of ground water occurs in many countries, including areas of the USA in which levels varied from less than 1 µg /L to over 3,100 µg /L depending on the area of the country. (Ayotte *et al.*,

2003; Burgess *et al.*, 2007; Nielsen *et al.*, 2010; Peters, 2008; Sanders *et al.*, 2012; Thundiyil *et al.*, 2007)

The primary route of exposure for the population at large is through ingestion of contaminated water or food. Daily intake in the general population is typically in the range of 20-300 µg /day (WHO, 2001). Occupational exposure through inhalation of particulates also occurs in a smaller subset of the population, such as those who prepare pressure treated wood or work in metal smelting.

Inorganic vs. organic arsenic was selected for this study because it is a commonly encountered environmental contaminant (National Toxicology Program, 2014), and it is considered more biologically important because it is readily absorbed by the body and highly reactive whereas organic compounds are poorly absorbed and considered relatively innocuous (Chung *et al.*, 2014). Additionally, arsenic can act as a co-carcinogen (Germolec *et al.*, 1997; Germolec *et al.*, 1998; Iarc Working Group, 2012; Rossman, 2003; Rossman *et al.*, 2001; Rossman *et al.*, 2002; Wang *et al.*, 2002), and as previously discussed, combinations of toxicants can have different effects than any single toxicant in isolation.

Arsenic levels in foods and beverages other than drinking water are not regulated in the U.S., although there are estimated Minimal Risk Levels (MRLs) for dietary intake. MRLs estimate the level of daily exposure over a certain period likely to cause no adverse, non-cancer health effects. According to the U.S. Agency for Toxic Substances and Diseases Registry (ATSDR), the MRL for chronic (greater than 365 days) arsenic exposure is 0.3 µg As/kg body weight per day (Wilson, 2015). MRLs consider only non-

cancer health effects in the most sensitive populations. Because cancer effects are not considered, the possibility remains that chronic exposure to even very low concentrations of these toxicants increases the risk of cancer development in susceptible individuals, and it is estimated by some that greater than 3 million Americans are exposed to levels of iAs above the EPA's maximum contaminant level of 10 µg /L (Naujokas *et al.*, 2013).

Arsenic intake through diet has historically been considered generally low and of relatively minor concern, relative to exposure through drinking water, with regard to carcinogenesis (Iarc Working Group, 2012; WHO, 2001). Foods with the highest concentrations of arsenic are seafood, rice (including rice cereal), mushrooms, and poultry (National Toxicology Program, 2014). Several recent studies have shown that arsenic exposure can exceed proposed limits in populations that consume a large amount of rice, especially if the rice is grown in As contaminated water or if they consume multiple arsenic-contaminated foods or beverages (Adomako *et al.*, 2009; Meharg *et al.*, 2008; Rahman *et al.*, 2011; Stone, 2008; Zhu *et al.*, 2008). Arsenic concentration varies based on where it is grown and the variety of rice (Rahman, et al., 2011; Signes-Pastor *et al.*, 2016; Wilson, 2015). In addition, iAs contamination has been documented in food products for children that are derived from rice (Meharg, et al., 2008; Wilson, 2015).

Arsenic is a known carcinogen affecting skin, lung, liver, urinary bladder, and other sites (Chen *et al.*, 1988; National Toxicology Program, 2014; Smith *et al.*, 1992). For example, statistically significant increases in urinary bladder cancer have been associated with exposure to 50 µg/L iAs exposure levels in drinking water

(Christoforidou *et al.*, 2013). In addition to cancer effects, chronic arsenic exposure has been associated with renal damage as measured by proteinuria (Chen *et al.*, 2011), and in some studies, with increased risk of developing CKD (Diyabalanage *et al.*, 2017; Hsu *et al.*, 2017; Orr *et al.*, 2017; Zheng *et al.*, 2015). As previously mentioned, CKD is of concern because its incidence is increasing, and diagnosis is usually made late in the course of disease, when little can be done to ameliorate its effects. While kidney damage and tumors of the kidney and urinary bladder are associated with arsenic exposure, animal models for these cancers are lacking (National Toxicology Program, 2014).

While several animal models for CKD exist, because of the diversity of initiating causes of CKD and because the models are often strain, age, and/or sex dependent, no single model can exactly mirror human CKD (Rabe *et al.*, 2016; Yang, et al., 2010). Many factors must be taken into consideration when choosing a rodent model for kidney disease, including the previously mentioned strain, sex, and age of the rodents to be used as well as mechanism of damage, genetic modifications, and genetic background of transgenic models. For example, C57BL/6 mice, the most widely used strain, is relatively resistant to hypertension (a factor in the progression of kidney disease) and proteinuria (one measure of kidney damage).

A possible reason for the lack of rodent models that approximate human development of kidney disease and cancers after toxicant exposure that can be addressed is that mice are extremely efficient at removing xenobiotics, including heavy metals like arsenic, from renal tubules via the multidrug resistance pump. Mice have two genes (*Mdr1a* and *Mdr1b*) coding for the equivalent of the human multidrug resistance (MDR)

pump that is involved in extruding various substances from renal tubular cells. Because mice are amenable to genetic manipulation, the equivalent multidrug resistance genes in mice can be knocked out to study how this affects xenobiotic processing. When *Mdr1a/1b*$^{-/-}$ mice were exposed to arsenic in one study, the mice had increased sensitivity to acute arsenic toxicity compared to wild-type mice, and they had higher arsenic accumulation in tissues including the kidney (Liu *et al.*, 2002b), better modeling that observed in human tissues.

1.5. Genetic Heterogeneity in Toxicological Testing

As previously stated, toxicological testing has generally been performed in genetically homogeneous rodent populations. A major advantage of this approach is that these models provide a well-defined, stable population for study that is readily available and has a large amount of historical data established. They are essentially a defined reagent, allowing reproducibility. On the other hand, one major disadvantage of this approach is that use of a genetically homogeneous study population may mask detection of variable responses to toxicants due to genetic variability.

Several studies have illustrated this problem. A study of acetaminophen using 35 different inbred strains of mice against the NTP-preferred B6C3F1 hybrid showed that there was great variability in hepatotoxicity among strains (Harrill *et al.*, 2009). Thus, using a single mouse strain to assess hepatotoxicity might result in erroneous conclusions about the safety of this drug. Other studies of environmental toxicants

(Koturbash *et al.*, 2011; Nguyen *et al.*, 2017; Yoo, et al., 2015b) and nanoparticles (Scoville *et al.*, 2015) have found similar differences in response among strains of mice.

This study examines how chronic, low-dose exposure to mixtures of toxicants contributes to the development of adverse health outcomes in a genetically heterogeneous population and the genetic factors that influence susceptibility to these effects. This work is intended to provide a foundation for development of a model that is predisposed to the development of adverse renal health effects after exposure to our toxicants of interest: TCE and inorganic arsenic. With this information, the genetic factors influencing the susceptibility may be identified, and these genetic factors may provide targets for future research, not just in toxicology, but also in the development of CKD or cancer susceptibility.

2. RENAL TUBULAR DAMAGE BUT NOT TUMORIGENESIS RESULTS FROM LONG-TERM COMBINATORIAL EXPOSURE TO TRICHLOROETHYLENE AND INORGANIC ARSENIC IN GENETICALLY HETEROGENEOUS MICE DEFICIENT FOR MULTI-DRUG RESISTANCE

2.1. Overview

Trichloroethylene (TCE) and inorganic arsenic (iAs) are environmental contaminants that can target the kidney. Chronic exposure to TCE is associated with increased incidence of renal cell carcinoma, while co-exposure to TCE and iAs likely occurs in exposed human populations, such as those near Superfund sites. In order to better understand the kidney health consequences of TCE and/or iAs exposure, a genetically heterogeneous mouse population derived from FVB/N and CAST/EiJ mouse strains and deficient for multidrug resistance genes (*Abcb1a*^{tm1Bor}, *Abcb1b*^{tm1Bor}) was chronically exposed for 52-weeks to varying concentrations of TCE and iAs. Although no exposure group resulted in primary renal cell tumors, kidneys from exposed mice did have significant increases in histologic evidence of renal tubular disease with each toxicant alone and with combined exposure. However, no increase in tubular disease was observed with combination exposure compared to single toxicant exposure. While this model more accurately reflects human exposure conditions, development of primary renal tumors observed in humans following chronic TCE exposure was not reproduced, even after inclusion of the co-carcinogenic iAs in this model.

2.2. Introduction

Trichloroethylene (TCE) is a chlorinated solvent that has been widely used as an industrial degreaser and dry cleaning agent in the past. Due to its wide use and improper disposal, TCE has been found at the majority of the current and proposed National Priority List (Superfund) sites (Chiu *et al.*, 2006), and is the most commonly reported organic groundwater contaminant (National Research Council (U.S.) Committee on Human Health Risks of Trichloroethylene., 2006). Exposure to TCE has been associated with a variety of cancers and disorders, including renal cancers and renal injury (Liu, et al., 2010; Radican, et al., 2006). The most recent estimates from the National Cancer Institute (NCI) are that almost 64,000 new cases of kidney and renal pelvis cancer will be diagnosed in 2017, which accounts for 3.8% of all new cancer cases in the United States and an annual incidence of 15.6 per 100,000 people (Bakke, et al.). In some areas of the country, often overlapping with Superfund sites, renal cancer incidence is higher than the national average. One such location is Onslow County, North Carolina, home to a Superfund Site (Camp Lejeune) contaminated with trichloroethylene (TCE). According to the State Cancer Profiles from the Center for Disease Control and Prevention (CDC) and NCI, Onslow County had an annual renal cancer incidence of 21.3 per 100,000 from 2008-2012, well above the national average (Centers for Disease Control and Prevention).

There has recently been increased interest and study on the effects of chemical or toxicant mixtures on health outcomes (Kapraun, et al., 2017; Pollock, et al., 2017), particularly for toxicants like TCE whose effects on disease severity and outcome may

be mediated by co-exposures in the environment. Inorganic arsenic (iAs) is a prime candidate for studying the interactions of multiple toxicants in a more controlled setting than is possible using human retrospective studies.

Inorganic arsenic is a commonly encountered environmental contaminant found in soil and groundwater secondary to anthropogenic activities including mining, farming, and fossil fuel combustion (National Toxicology Program, 2014; Naujokas, et al., 2013). Exposure to iAs is associated with adverse health effects and various cancers in humans. For example, statistically significant increases in urinary bladder cancer have been associated with exposure to 50 μg/L iAs exposure levels in drinking water (Christoforidou, et al., 2013), and chronic exposure to arsenic is associated with the development of chronic kidney disease in humans (Diyabalanage, et al., 2017; Hsu, et al., 2017; Zheng, et al., 2015). In addition to drinking water, iAs exposure may occur through foods and processed beverages such as rice grown in areas with high iAs levels (Davis *et al.*, 2012; Gilbert-Diamond *et al.*, 2011; Jackson *et al.*, 2012; Meharg, et al., 2008; Naujokas, et al., 2013). Consequently, increased concentrations of iAs in rice may lead to increased exposure in individuals consuming rice from regions with high levels of soil or water iAs contamination (Stone, 2008). Chronic exposure to iAs at levels higher than the current EPA maximum contaminant level (10 μg/L) are estimated to occur in greater than 3 million people in the United States (Naujokas, et al., 2013), and it is reasonable to expect that at least some of the people exposed to iAs will also be exposed to other environmental toxicants.

Human epidemiological studies on TCE are frequently confounded by exposure to other toxicants and multiple cancer risk factors, and they are limited by lack of detailed information regarding route, duration, dose, and cumulative exposure to the toxicant of interest. While these studies of exposed human populations provide the strongest link between toxicant exposure and human disease due to those toxicants, use of appropriate mouse models can provide evidence of toxicity and strengthen the case for human health effects.

Toxicological studies classically use a single strain of rodent. The National Toxicology Program (NTP) historically used the B6C3F1 hybrid mouse and inbred Fisher 344 rat (until 2006), although currently the Harlan Sprague Dawley rat is the standard rat strain used in NTP carcinogenicity studies (King-Herbert, et al., 2010), and some studies have used other strains of rat. For example, an NTP carcinogenicity study on TCE reported in 1988 (TRS 273) utilized four different strains of rat. Due to the long history of using standard NTP models in carcinogenicity studies, there is an extensive knowledge base, including expected background lesions, and this facilitates cross study comparisons, another advantage of the classical toxicological approach.

NTP carcinogenesis studies for TCE used gavage delivery in corn oil with doses determined based on results of a 13-week NTP study (Chhabra *et al.*, 1990), which in turn were determined from previous TCE studies (National Cancer Institute, 1976). These studies did not reference human exposure data as a basis for dosing in experimental animals. In the NTP carcinogenesis study on TCE, a single dose level was chosen for TCE in mice based on the results of a previous 13-week toxicity study and

not based on known environmental exposure levels. In this study, survival of TCE-exposed male mice was lower than that of controls, and cytomegaly was reported in male and female TCE-exposed mice but none of the vehicle-exposed mice. Increased incidence of hepatocellular tumors was observed in mice, but no renal tumors were observed.

For arsenic, classification as a carcinogen is based on evidence of carcinogenicity in humans where it is known to cause increased incidence of cancers of the urinary bladder and kidneys, as well as other organs depending on route of exposure (National Toxicology Program, 2014). In carcinogenicity studies of arsenic utilizing multiple routes of exposure and multiple animal species, no tumors were detected or the results were inconclusive in most cases, although increases in urinary bladder cancers and liver adenomas were observed in some rat studies, and increased lung tumor number and size was observed in the A/J strain (Arnold *et al.*, 2006; Cui *et al.*, 2006; Iarc Working Group, 2012; National Toxicology Program, 2014; Shen *et al.*, 2003; Wang, et al., 2002).

While the classic NTP approach has some advantages, it also has substantial limitations. Due to the lack of genetic diversity in the models, these models do not capture the variation in response to toxicants that is due to genetic heterogeneity in the humans they seek to model. They also have the potential to incorrectly predict the toxicity of an agent, because different strains of mice may have differing sensitivity to toxicants, resulting in over or underestimation of the actual toxicity of an agent.

Evaluation of human health outcomes due to chronic low dose toxin exposure has been limited by scarcity of research closely mimicking the chronic, low-dose exposure to mixtures of toxicants to which humans are exposed. Classical toxicology studies investigate one potential toxicant at a time. While this is in keeping with the scientific principle of studying only one variable at a time, it does not mirror natural exposure, and thus these studies are limited in their ability to predict or model human health outcomes. Experimental paradigms that combine human-relevant, chronic, low dose exposures to mixtures of environmental toxicants and genetically heterogeneous populations as well as nutrition designed to mimic that of exposed human populations have the potential to improve the human health relevance of toxicological research. In this study, we used two common environmental toxicants in doses designed to mimic those identified in the environment, used a genetically heterogeneous population of mice to capture genetic variability, and fed a diet whose nutrient profile is similar to the typical western diet to better model the nutritional environment in which these toxicants would have their effects in the US.

2.3. Materials and Methods

2.3.1. Animals

All housing conditions and procedures were approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee. Previous work in this laboratory, when at NSCU, established a unique F_3 mouse population derived from two phylogenetically distant inbred mouse strains. The *Mus musculus domesticus*

inbred strain, FVB/N-*Abcb1a*^{tm1Bor}, *Abcb1b*^{tm1Bor}, has a mutation of the multi-drug resistance (MDR) transporter genes *Abcb1a* and *Abcb1b*, resulting in loss of function of the MDR transporter. Mice have an especially active MDR system, while *Mdr1a/1b*$^{-/-}$ mice exposed to arsenic have increased sensitivity to acute arsenic toxicity compared to wild-type mice, and had higher arsenic accumulation in tissues including the kidney (Liu *et al.*, 2002a), better modeling that observed in human tissues. The *M. musculus castaneous* inbred strain, CAST/EiJ is a wild-derived strain that is genetically distinct from the FVB/N strain with a highly divergent polymorphism profile.

Breeding of mice was performed in house at the NCSU Biological Resource Facility. The breeding colony and study mice were maintained in a temperature controlled environment at 21+/- 2°C on a 12 hour light and 12 hour dark schedule. Female FVB/N-*Abcb1a*^{tm1Bor}, *Abcb1b*^{tm1Bor} mice (Taconic Biosciences) were crossed with male CAST/EiJ mice (Jackson Laboratory) to create an F1 population that was intercrossed to create an F2 population. Because only one-quarter of mice in this generation were expected to carry the double *Abcb1b-/-* knockout and a large number of mice with this genotype were required for the study population, F2 mice were genotyped to identify mice that carried the double knockout of the MDR transporter (Schinkel *et al.*, 1997).

These mice were then intercrossed to produce the study population of F3 mice homozygous for *Abcb1a*^{tm1Bor}, *Abcb1b*^{tm1Bor} (Figure 2.1).

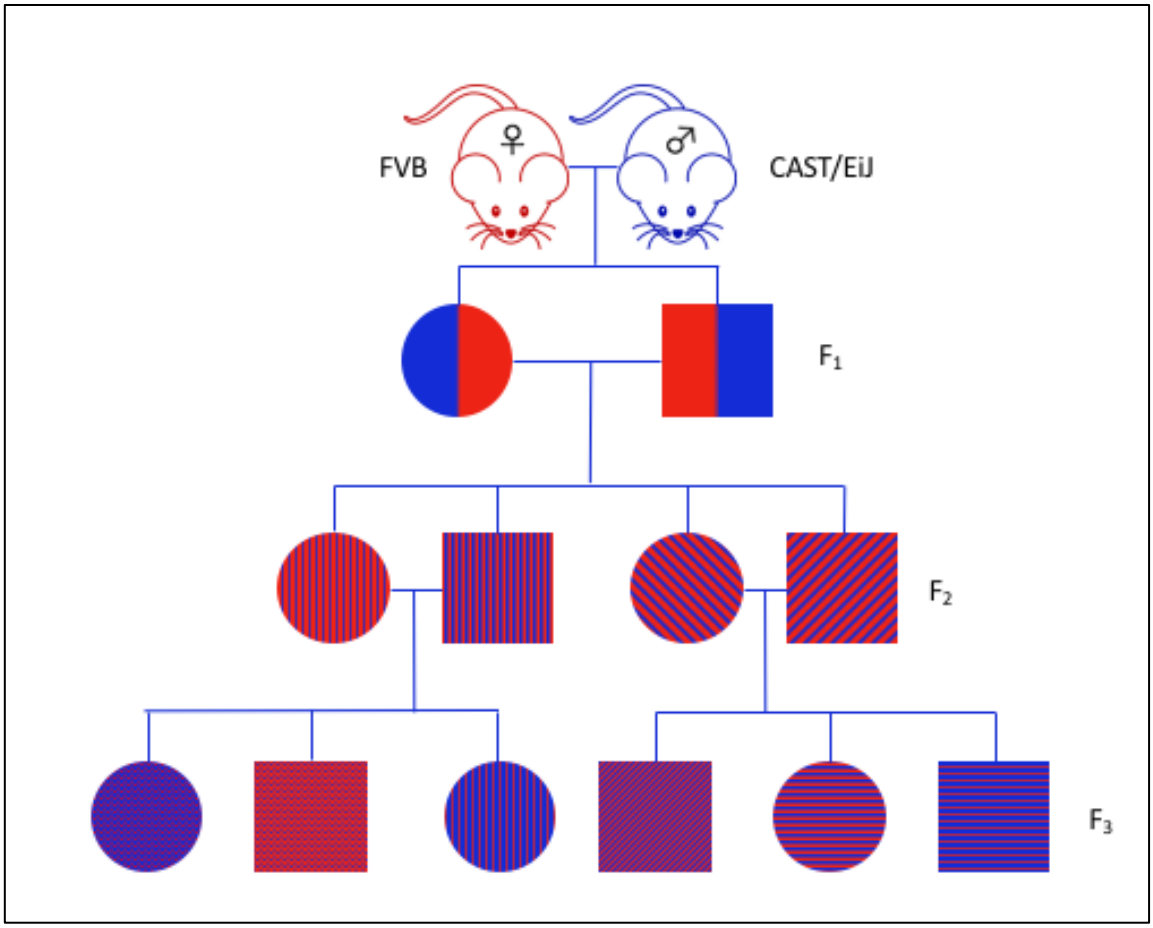

Figure 2.1 Breeding Plan.
Female FVB/N-*Abcb1a*^{tm1Bor}, *Abcb1b*^{tm1Bor} mice were mated to male CAST/EiJ mice. F1 progeny were intercrossed to produce an F2 population. To obtain an adequate number of double knockout mice to enter the study, F2 mice were genotyped and intercrossed to maximize the number of F3 mice with the *Abcb1/Abcb1b* double mutant genotype.

One hundred F3 mice (fifty males and fifty females) were randomly assigned to each of nine exposure groups with one same-sex pup per litter per group to eliminate

litter effects and facilitate detection of adverse health outcomes. Exposure dosages were calculated as dose equivalents based on human exposure data, and low and high doses were selected to be no more than 2 to 6-fold different than actual human exposures. For TCE, these values included well water measurements of TCE for the high dose and data from the National Health and Nutrition Examination Study (NHANES) for the low dose (National Research Council (U.S.) Committee on Human Health Risks of Trichloroethylene., 2006). Doses for arsenic were based on the World Health Organization (WHO) limit for the low dose and a calculation of arsenic in rice combined with average daily intake for the high dose (Stone, 2008). Exposure groups were designed to include no, low, and high doses of each toxicant and all possible combinations of these categories (Table 2.1).

Table 2.1 Toxicant Dose Groups

Group	Dose Ratio (iAs:TCE)	iAs Concentration (μg/kg food)	TCE Concentration (ppb in water)
1	None : None	0	0
2	None : Low	0	5
3	None : High	0	2850
4	Low : None	10	0
5	Low : Low	10	5
6	Low : High	10	2850
7	High : None	150	0
8	High : Low	150	5
9	High : High	150	2850

2.3.2. Toxicant Exposure

Mice, group housed in single sex cages, had *ad libitum* access to food and water. F3 generation mice were weaned at post-natal day 21 onto AIN-93M standard diet (Envigo-Teklad Diets). At 6-weeks mice were switched from AIN-93M diet onto an American-style diet (Envigo-Teklad Diets) that was designed to be similar to the typical American diet with increased kcal from fat, skewed omega 6 to omega 3 fatty acid ratio, and deficient folic acid compared to the AIN-93M standard diet. Mice were allowed to acclimate to the American diet for 10-14 days before being assigned to a specific exposure group. Entry dates into specific treatment cohorts were staggered to minimize confounding by calendar date of procedures, with 200-300 F3 mice entering the study

every four to five weeks over a 16-week period. TCE (0, 5, or 2850 ppb) was added to purified drinking water, prepared fresh weekly, and administered in UV-light protected bottles to prevent degradation. iAs (0, 10, or 150 μg/kg as sodium arsenite) was mixed into a custom high fat American diet (Envigo-Teklad Diets). All food was replaced weekly and stored at 4°C in vacuum-sealed bags to prevent oxidation. Mice were maintained on their assigned treatment group for the 52-weeks of toxicant exposure. All sample collections and measurements were performed during narrow time windows to minimize circadian effects.

2.3.3. Sample Collection and Histopathologic Examination

Following 52 weeks of toxicant exposure, mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. At necropsy, kidneys and other organs (liver, lung, heart, and any organs with abnormal findings) were removed, weighed, and examined for gross abnormalities. Each kidney was halved longitudinally. One half of each kidney was fixed in 10% neutral buffered formalin for 24 hours, transferred to 70% ethanol, then routinely processed and paraffin embedded. The remaining half was flash frozen in liquid nitrogen for long-term storage. Five 5μm-thick serial sections were obtained and the first, third and fifth of these were hematoxylin and eosin (H&E) stained for histopathological examination. Light microscopic examination of kidney slides was performed by a board certified anatomic pathologist (AP). Each slide was randomly assigned a new identifier to mask exposure group from the pathologist. The first, third and fifth slides for each individual were examined for

neoplasia or preneoplastic changes. Following this initial examination, one representative slide from each individual was examined and scored for histologic evidence of renal disease.

2.3.4. Statistical Analysis

All statistical analyses of tubular disease scores were performed using JMP 13.0. Graphs were built in Prism 7 (Mac version 7.0c). Data having non-normal distributions were analyzed using the Kruskal-Wallis test. Post-hoc testing was performed using the Dunn method for multiple comparisons, which utilizes the Bonferroni adjustment to correct for multiple comparisons. The control group for statistical analyses was defined as the No iAs/No TCE group.

2.4. Results

2.4.1. Survival is Not Affected by Exposure Group

As previously described, one hundred F3 mice (fifty males and fifty females) were randomly assigned to each of nine exposure groups with one same-sex pup per litter per group to eliminate litter effects and facilitate detection of adverse health outcomes. Toxicant exposure occurred for 52 weeks.

No significant differences were found in a Kaplan-Meier survival analysis through the end of the study between any exposure group and the No iAs/No TCE (control) group . Log-rank testing of survival showed a significant difference among the groups ($p=0.047$), but subsequent multiple comparisons showed no difference between

the control group and any of the treatment groups. Overall, male mice experienced greater mortality, regardless of group, by the end of the study (Tables 2.2 and 2.3).

Table 2.2 Deaths per Group During Study Period

	No As/No TCE	**No As/Low TCE**	**No As/High TCE**	**Low As/No TCE**	**Low As/Low TCE**	**Low As/High TCE**	**High As/No TCE**	**High As/Low TCE**	**High As/High TCE**
19 weeks	3	0	6	0	2	2	1	0	0
35 weeks	11	10	18	18	11	8	10	7	15
52 weeks	29	30	31	31	39	25	26	41	39
Total deaths	43	40	39	49	52	35	37	48	54

Table 2.3 Male and Female Deaths per Group

	Male	Female	**Male**	Female	**Male**	Female	**Male**	Female	**Male**	Female	**Male**	Female	**Male**	Female	**Male**	Female	**Male**	Female
19 week	3	0	0	0	5	1	0	0	2	0	2	0	1	0	0	0	0	0
35 week	11	0	10	0	18	0	17	1	8	3	7	1	2	8	6	1	12	3
52 week	17	12	22	8	8	7	23	8	23	16	16	9	17	9	23	18	27	12
Tot	31	12	32	8	31	8	40	9	33	19	25	10	20	17	29	19	39	15

2.4.2. Exposure is Associated with Renal Tubular Disease

While no changes in overall survival to the end of the study were observed, significant differences between dose groups in tubular disease score as measured by histologic examination were observed. Lesions included in the evaluation were scored based on numerical criteria (Table 2.4; Figure 2.2). Diagnoses were based on criteria published as part of the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) Project (Frazier *et al.*, 2012). In cases of potential neoplasia of any type, histologic sections of additional organs (lung, liver, or heart, as available) from the same individual were examined for evidence of metastasis or disseminated disease. Tubular disease lesion scores were determined by summing the individual scores in each category, resulting in a total score for each animal between 0 and 20.

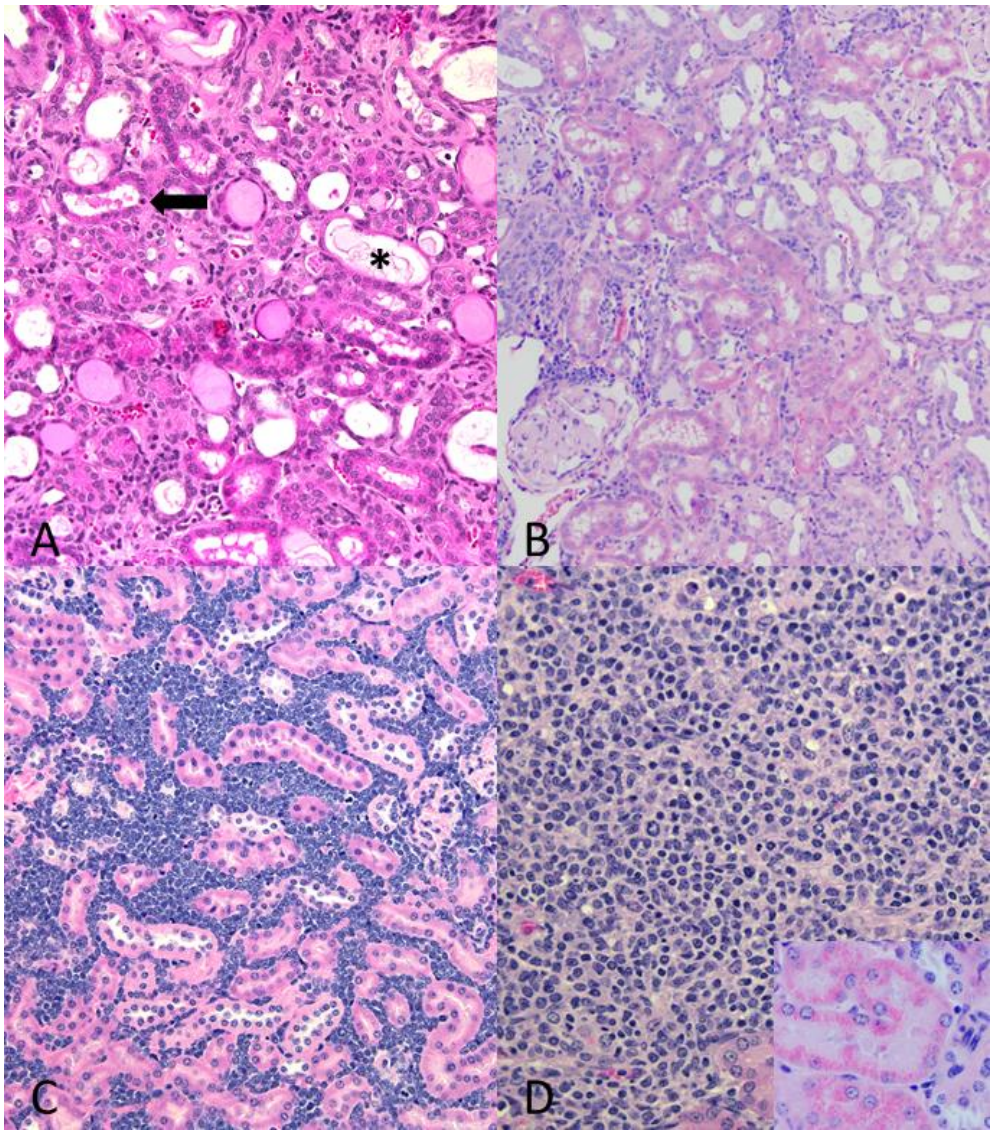

Figure 2.2 Examples of Renal Pathology.
(A) Kidney with tubular dilation (asterisk), degeneration and regeneration. One tubule has a necrotic tubular epithelial cell (arrow). (B) Kidney. Example of chronic progressive nephropathy lesions. Areas of relatively well demarcated tubular change consisting of basophilic tubules with thickened basement membranes. A glomerulus in the section is expanded by presumed amyloid. (C) Kidney with lymphoma. (D) Kidney with histiocytic sarcoma. Hyaline droplets are a recognized lesion observed in renal tubular epithelium of mice with histiocytic sarcoma (inset, lower right).

Table 2.4 Tubular Disease Scoring Criteria

Score	Chronic progressive nephropathy (CPN)	Tubular degeneration +/- regeneration	Tubular dilation	Karyomegaly	Tubular single cell necrosis	Tubular epithelial microvesicles
0	None	None	None	None	Absent	None
1	Rare affected tubules	Rare tubules with evidence of degeneration or regeneration not associated with lesions of CPN	Rare tubules with lumens ≥50% of total tubule diameter	Rare tubular cells with nuclei ≥2X the size of normal nuclei	Present	Present in rare tubules
2	Few foci of affected tubules	Few tubules with evidence of degeneration or regeneration not associated with lesions of CPN	Few tubules with lumens ≥50% of total tubule diameter	Few tubular cells with nuclei ≥2X the size of normal nuclei	N/A	Present in few tubules
3	Many foci of affected tubules	Many tubules with evidence of degeneration or regeneration not associated with lesions of CPN	Many tubules with lumens ≥50% of total tubule diameter	Many tubular cells with nuclei ≥2X the size of normal nuclei	N/A	Present in many tubules
4	Most tubules affected	Most tubules have evidence of degeneration or regeneration not associated with lesions of CPN	Most tubules have lumens ≥50% of total tubule diameter	N/A	N/A	Present in most tubules

Comparisons were made between each treatment group and the unexposed group. As compared to the No iAs/No TCE group (mean score = 1.53), increases in mean tubular disease scores were detected in the No iAs/Low TCE (mean score = 3.47, $p<0.0001$), No iAs/High TCE (mean score = 2.83, $p=0.0184$), Low iAs/No TCE (mean score = 3.76, $p<0.0001$), Low iAs/High TCE (mean score = 3.53, $p<0.0001$), High iAs/No TCE (mean score = 4.00, $p<0.0001$), and High iAs/High TCE (mean score = 3.48, $p<0.0068$) groups (Figure 2.3). In addition, increases in tubular disease scores were observed in animals exposed to TCE alone, iAs alone, and in those exposed to both toxicants in combination, although there was no increase in average severity of disease in those with combination exposure to TCE and iAs compared to those with single toxicant exposure (Figure 2.4).

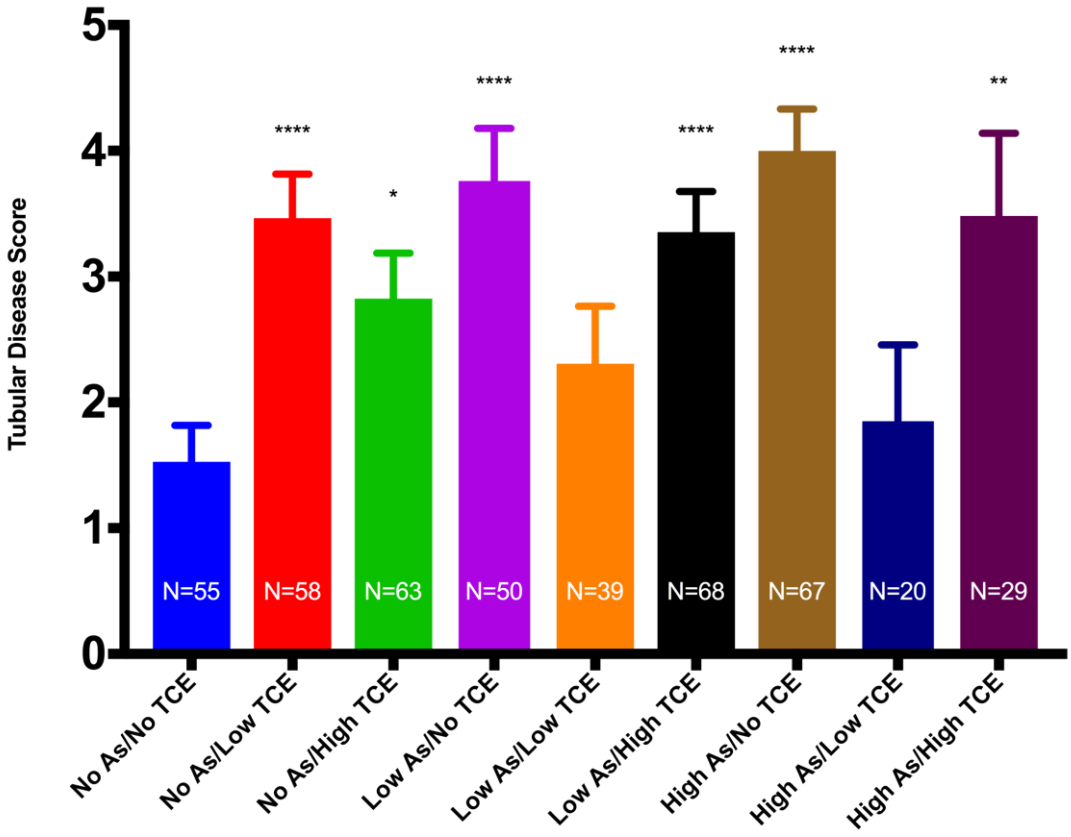

Figure 2.3 Tubular Disease Score by Exposure Group.
Mean (+SE) tubular disease score in each dose group following 52-week exposure to TCE, iAs, or both. No As/No TCE group was used as control for comparisons: **** $p<0.0001$; ** $p=0.0068$; * $p=0.0184$.

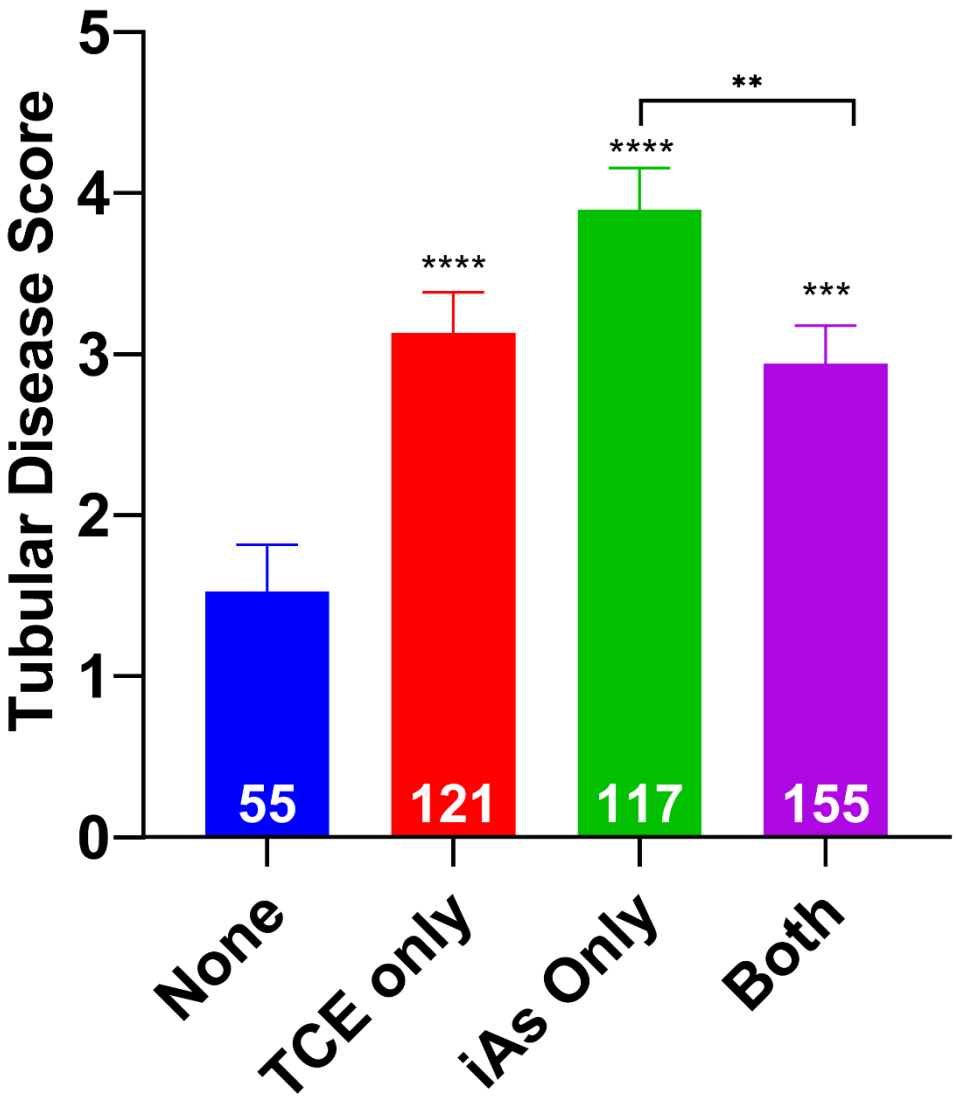

Figure 2.4 Tubular Disease Score by Toxicant Exposure Status.
Mean (+SE) tubular disease score by toxicant exposure or co-exposure following 52-week exposure to TCE, iAs, or both. No Toxicant group used as control for comparisons: **** $p<0.0001$, *** $p=0.0003$. Significantly higher tubular disease scores are observed for all toxicant exposed groups, but the tubular disease score is significantly decreased in the combination exposure group compared to the iAs only group.

2.4.3. Exposure and Sex Influence of Non-Renal Neoplasia

No tubular epithelial neoplasms or pre-neoplastic changes of tubular epithelium were observed in the examined sections. Seven instances of infiltrative round cell neoplasms (histiocytic sarcoma and presumed lymphoma) affecting the kidney were identified (Table 2.5). Of the seven round cell neoplasms identified in the kidneys, six were in female mice, and four of the seven were identified in mice with iAs exposure but no TCE exposure (Figure 2.3).

Table 2.5 Renal Neoplasia

Dose Group	Sex	Tumor Type	Other Affected Organs
No As/Low TCE	F	Lymphoma	None detected
Low As/No TCE	F	Histiocytic sarcoma	Liver, Lung
Low As/No TCE	F	Histiocytic sarcoma	Liver
Low As/No TCE	F	Lymphoma	Liver, Lung
Low As/High TCE	F	Lymphoma	Liver
High As/No TCE	M	Lymphoma	Liver, Lung, Cranial mediastinal mass
High As/High TCE	F	Histiocytic sarcoma	Lung, Lymph node, Cranial mediastinal mass

2.5. Discussion

Developing an accurate rodent model of the development of human TCE-associated renal tumors, specifically clear cell renal cell carcinoma (ccRCC), has been challenging. Toxicological studies generally evaluate toxicants in isolation and in

genetically homogeneous populations of rodents, even though this does not reflect the genetic variability of exposed human populations or the typical scenario of exposure in which multiple toxicants are often encountered together. Response to toxicant exposure is governed by many factors including intrinsic (genetic and epigenetic variation, age and life stage, sex) and extrinsic factors (co-exposures to other toxicants, nutritional state, stressors, dosage, co-morbidities) (Zeise *et al.*, 2013).

It is becoming increasingly clear that toxicological studies must address the effects of genetic variability in the human population and the range of sensitivity to toxicity that may result from this inherent variability. In addition, experimental paradigms that assess and control for as many of the intrinsic and extrinsic factors as possible that affect health outcomes are also needed. In human epidemiological studies, there is often a lack of critical detailed information about precise exposure levels, co-exposures, time period of exposure, or other risk factors.

In addition to including genetic heterogeneity and nutrition modeled on the typical American diet, our study included a second common environmental toxicant, iAs, to investigate the potential interactions of two commonly occurring toxicants. Arsenic is a known renal toxicant and urinary system carcinogen when exposure occurs through drinking water (Christoforidou, et al., 2013), and it is also known to accumulate and become concentrated in plant-based food products grown in arsenic-containing water (Davis, et al., 2012; Gilbert-Diamond, et al., 2011; Jackson, et al., 2012), which provides another avenue for co-exposure to this toxicant.

In this study, we analyzed long-term exposure to TCE alone or in combination with iAs. Previous studies have shown large variability among strains in metabolism and response to TCE (Cichocki *et al.*, 2017), suggesting long-term TCE exposure in genetically heterogeneous mice could lead to ccRCC. Surprisingly, there were no differences in survival among dose groups; even the control group had a relatively large mortality (43% before the end of the study). One factor in lack of survival differences is that, in the present study, only renal health was examined. Both TCE and iAs are known to have liver and other health effects (Liu, et al., 2010; Singh *et al.*, 2007; Wartenberg, et al., 2000), which may have contributed to the similar mortality in the toxicant-exposed groups. Another factor to consider is conspecific aggression, relevant to control and exposed groups. Aggression among laboratory mice, particularly male mice, is a known issue and has proven difficult to reduce in group housing. Many variables are involved in aggression between laboratory mice, including but not limited to size of housing, number of animals per cage, bedding, shelters, temperature, strain, and stress (Weber *et al.*, 2017). Efforts were made in the present study to limit the effects of aggression, such as the use of larger than standard cages and limiting exposure to unfamiliar animals. Even so, some actions that may limit aggression, for example keeping littermates together, could not be implemented in the present study design that was chosen to limit litter effects.

Significant differences in histologic evidence of renal tubular disease were observed among the different treatment groups, including increases in tubular disease scores between animals exposed to TCE alone, exposed to iAs alone, and those exposed

to both toxicants, although no difference in severity was observed in those co-exposed, contrary to our expectations. It is possible that damage caused by one toxicant was not increased by exposure to a second renal toxicant as the cells already damaged to the point of requiring regeneration could not be further damaged by the second toxicant. Although the study was designed to increase renal carcinogenic potential, primary renal tubular cell neoplasms failed to develop.

Limitations of animal models for ccRCC due to TCE exposure, including in the present study include that the rate of ccRCC in humans is relatively low. Because the annual incidence rate in humans is low even in exposed populations, if mice developed ccRCC at a similar rate to exposed humans, approximately 5000 animals would be needed to detect one case. This makes studying these tumors difficult even in a rodent model, due to the large number of individuals required to detect the formation of these tumors. Another factor complicating the modeling of this cancer in rodents is that it requires long periods of time for tumors to develop, increasing the incidence of age-associated sporadic lesions, such as chronic progressive nephropathy (CPN), that complicate histopathologic interpretation. Further, rodents, especially mice, have a higher capacity for metabolism of TCE than humans, (Lash *et al.*, 2000a), making the toxic metabolite profile and subsequent organ system pathology potentially different from that seen in humans.

Rats have been the preferred rodent model used to study the effects of TCE exposure, but development of CPN is nearly certain in rats as they age, and the regenerative response in CPN can be quite florid, mimicking pre-neoplastic or neoplastic

changes and making interpretation of renal lesions and their significance to risk of tumor development in humans difficult. While mice also develop CPN with age, the incidence and severity of lesions is generally less than with rats.

In addition to the limitations of the model discussed above, limitations of this study include that mice are not typically used to model renal cancer in toxicology studies. Mice have not been documented to develop ccRCC after chronic exposure to TCE; however, data on co-exposures to toxicants are lacking, and given the information about arsenic's role as a potential co-carcinogen, it was theorized that inclusion of this second toxicant could lead to development of renal tumors in mice. Another limitation is that there is limited historical data on background lesions and expected pathology in the strains of mice used in this study, and no information is available regarding the crosses involving these mouse strains. This can complicate interpretation of pathological findings

Despite these limitations, the authors believe that the current study used a model more accurately reflecting human exposure conditions by including multiple toxicants, environmentally relevant concentrations, a genetically heterogeneous population, and a long period of exposure. Despite the more accurate modeling of human exposure conditions, the primary renal tumor development observed in humans following chronic TCE exposure was not observed in this study. Renal tubular disease did occur both in single TCE- and iAs-exposed groups, as well as combination-exposed groups, although the magnitude of tubular diseased was not different between single and combination exposed groups.

3. TOXICANT EXPOSED AND UNEXPOSED MALE MICE HAVE HIGHER RENAL DAMAGE BUT RENAL DAMAGE IS NOT INCREASED BY COMBINATION EXPOSURE TO TRICHLOROETHYLENE AND INORGANIC ARSENIC

3.1. Overview

Trichloroethylene (TCE) and inorganic arsenic (iAs) are common environmental contaminants that can cause renal disease including renal cell carcinoma with chronic exposure to TCE and increased incidence of chronic renal disease from either toxicant. Co-exposure to TCE and iAs likely occurs in some human populations, such as those near Superfund sites. A genetically heterogeneous mouse population derived from FVB/N and CAST/EiJ mouse strains and deficient for multidrug resistance genes (*Abcb1a*^{tm1Bor}, *Abcb1b*^{tm1Bor}) was chronically exposed for 52-weeks to varying concentrations of TCE and iAs to better understand the effects of these toxicants on the development of renal disease following exposure, either in isolation or in combination. Kidneys from male mice had increased evidence of renal damage on histologic examination, urinalysis, and clinical chemistry. Similar to previously reported results, exposed mice had increased evidence of renal tubular disease with each toxicant alone and with combined exposure. However, no increase in renal disease was observed with combination exposure compared to single toxicant exposure. There was also no effect on urinary NGAL observed with toxicant exposure, and there was only moderate correlation between urinary NGAL and histopathologic indicators of renal disease.

3.2. Introduction

Kidney diseases are the 9th leading cause of death in the United States (Murphy SL, et al., 2017). The CDC estimates that 30 million people, or 15% of the United States population has CKD (Centers for Disease Control and Prevention, 2017). The kidney is responsible for, among other functions, clearance of waste products from normal body processes, maintenance of fluid volume within the body, and metabolism and excretion of drugs and environmental toxicants (Perazella, 2009).

Because the kidney is the primary eliminator of drugs and toxins, it is vulnerable to injury from these substances. However, there are many other factors that can increase susceptibility to renal damage, including intrinsic factors such as cardiovascular disease, diabetes mellitus, sepsis, liver disease, and genetic factors and extrinsic factors including environmental contaminants, natural environmental substances, and drugs. The development of kidney disease is most often multifactorial (Barnett *et al.*, 2018; Perazella, 2009), and the combination of these factors is thought to be responsible for much of the variability in kidney disease in response to exposure to renal toxicants.

Nephrotoxicity requires adequate exposure to an agent capable of causing kidney injury (Perazella, 2009). Depending on the degree of injury and the other contributing factors, renal injury may be followed by healing and re-establishment of renal function or permanent loss of nephrons and ongoing renal disease. (Barnett, et al., 2018) Regardless of the source of initial injury and other contributing factors, once a certain threshold has been passed, kidney disease is progressive with eventual development of chronic kidney disease (CKD) and end stage kidney disease. Because remaining

functional nephrons can compensate until about 75% of renal functional mass is lost, almost half of those with severe kidney disease are not aware that they have CKD. Due to the lack of clinical signs associated with earlier stages of kidney disease, and the lack of sensitive and specific diagnostic screening tests, CKD is often not diagnosed until later stages, when little can be done to treat the disease beyond dialysis and, eventually, renal transplant.

Renal cancers and CKD share some common risk factors, including obesity, smoking, and hypertension. Exposure to some environmental toxicants, including TCE and arsenic, has been established as a risk factor for the development of kidney cancer (National Center for Environmental Health, 2016). Recently, studies have linked CKD with environmental toxicants, especially with chronic cumulative exposure, although, as previously mentioned, CKD is multifactorial, and genetic susceptibility and other health conditions likely play a large role in the development of this disease (Kataria, et al., 2015; Soderland, et al., 2010).

As stated in Chapter 2, people are often exposed to mixtures of environmental toxicants, and it is therefore imperative that the effects of combination exposures to environmental toxicants on kidney disease and cancer are studied in a model that closely mirrors the conditions that humans are likely to encounter. Two common environmental toxicants, trichloroethylene (TCE) and inorganic arsenic (iAs), are the focus of this study.

Trichloroethylene is a common environmental toxicant that has been found in the majority of current and proposed National Priority List (Superfund) sites and is the most

commonly reported organic groundwater contaminant. Large numbers of people live in proximity to a Superfund site, although this does not necessarily increase the risk of adverse health effects due to differences in contaminants and containment at these sites. As previously stated, TCE exposure has been associated with the development of renal cell carcinoma (RCC) as well as renal injury (Environmental Protection Agency, 2001; Liu, et al., 2010; National Toxicology Program, 2014; Wartenberg, et al., 2000). Specifically, exposure to hydrocarbons including TCE has been associated with the development of CKD (Radican, et al., 2006; Ravnskov, 2000; Wedeen, 1992). Indeed, Radican *et al.* found that there was a 2-fold greater risk of developing end-stage kidney disease with TCE exposure.

Arsenic is another common environmental toxicant that can occur naturally in the environment or be an environmental pollutant due to human usage of this element in applications, including wood preservation, pesticides, mining, and pharmaceuticals. High levels of arsenic contamination of ground water occur in many countries, including areas of the USA. Chronic arsenic exposure has been associated with renal damage as measured by proteinuria (Chen, et al., 2011) and, in some studies, with increased risk of developing CKD (Orr, et al., 2017). As previously mentioned, CKD is of concern because it is common, and the diagnosis is usually made late in the course of the disease, when little can be done to ameliorate its effects. While kidney damage and tumors of the kidney and urinary bladder are associated with arsenic exposure, animal models for these cancers are lacking (National Toxicology Program, 2014).

Environmental exposure to various potential nephrotoxicants is a continuing problem (Perazella, 2009). Some of the potential nephrotoxicants include hydrocarbon solvents such as TCE and heavy metals such as arsenic. Studies of nephrotoxicants typically focus on a single agent. Such studies are necessary to determine specific mechanisms involved in toxicity, but they may not accurately reflect real-life situations in which multiple toxicants are encountered, comorbidities exist, and differing genetic susceptibility is in play (Barnett, et al., 2018).

The biochemical and molecular mechanisms of renal injury have been the focus of many studies. A recent review of mechanisms of acute kidney injury (AKI), which may progress to CKD, supports a concept called in-common mechanisms. The idea behind the in-common mechanism concept is that even though the agents causing kidney injury are different, they will induce similar mechanisms of action that result in kidney injury (Barnett, et al., 2018; Hultstrom *et al.*, 2018). Although there is evidence of common mechanisms at play, there is also evidence that differing etiologies can have unique features as well (Hultstrom, et al., 2018). Particularly in light of the possibility of combination exposures to toxicants, it is important to study how the different toxicants in combination may cause greater or lesser damage than in single exposure situations.

In this study, we utilized two common environmental toxicants at doses designed to mimic known human exposures to model combination environmental exposures. We also used a genetically heterogeneous population of mice to capture genetic variability, and we fed a diet whose nutrient profile is similar to the typical western diet to better model the nutritional environment in which these toxicants would have their effects in

the US. In this chapter we examine evidence of kidney disease induced by TCE and iAs via histopathologic examination of the kidney tissue, classical markers of kidney disease (urea nitrogen (BUN), creatinine, and proteinuria), and a more recent biomarker of renal injury, NGAL.

3.3. Materials and Methods

3.3.1. Animals and Toxicant Exposure

Briefly, as described in Chapter 2, a unique F_3 mouse population was derived from two phylogenetically distant inbred mouse strains: *Mus musculus domesticus* inbred strain, FVB/N-*Abcb1a*^{tm1Bor}, *Abcb1b*^{tm1Bor}, and *M. musculus castaneous* inbred strain, CAST/EiJ.

Mice, housed in groups of 10 in single sex cages, had *ad libitum* access to food and water. At post-natal day 21, mice were weaned onto AIN-93M standard diet (Envigo-Teklad Diets) and at 6-weeks were switched and acclimated to an American-style diet (Envigo-Teklad Diets) as previously described. Mice were assigned to a treatment group and maintained in this group for the duration of the toxicant exposure (52 weeks exposure). TCE (0, 5, or 2850 ppb) was added to purified drinking water, prepared fresh weekly, and administered in UV-light protected bottles to prevent degradation. iAs (0, 10, or 150 μg/kg as sodium arsenite) was mixed into a custom high fat American diet (Envigo-Teklad Diets). All sample collections and measurements were performed during a narrow time window (approximately 11:00 a.m. to 1:00 p.m.) to minimize circadian effects.

3.3.2. Sample Collection and Analysis

3.3.2.1. Serum

Blood collection was performed prior to the beginning of the toxicant exposure study as well as at intermediate times after 19 weeks and 32 weeks of toxicant exposure. At the conclusion of the 52-week toxicant exposure, mice were euthanized, and necropsy was performed. Immediately following euthanasia, whole blood was obtained via cardiac puncture. For all blood collections, blood was allowed to clot for up to 30 minutes at room temperature in tubes without any additive. The blood was then centrifuged at 10,000 x g for 10 minutes to separate serum from erythrocytes and leukocytes. Serum was transferred into fresh 1.5 mL clear Eppendorf polypropylene tubes and stored at -80°C for seven years for future analysis. Only serum from the terminal collection was analyzed for the present study due to low sample volumes and to facilitate detection of maximal effects.

3.3.2.2. Urine

Urine was collected prior to the beginning of the toxicant exposure study, at intermediate times after 19 weeks and 32 weeks of toxicant exposure, and prior to euthanasia at the conclusion of each mouse's participation in the study by use of metabolic cages. Mice were individually housed in metabolic cages (Hatteras Instruments Inc., Cary NC) for 16 hours with *ad libitum* access to drinking water. Cages collect voided urine and exclude feces via a funnel that channels urine into a 2 mL polypropylene collection tube. The collection tube is maintained at 1° to 6°C for the

duration of collection. Urine was centrifuged at 10,000 x g for 5 minutes to separate particulate matter, 30 μL aliquots were made into fresh clear polypropylene tubes, and aliquots and original sample tube were stored at -80°C for future analysis.

3.3.2.3. Histopathology

As previously described, at the conclusion of the 52-week toxicant exposure, mice were euthanized and necropsy was performed. Each kidney was halved longitudinally, and one half of each kidney was fixed in 10% neutral buffered formalin for 24 hours, transferred to 70% ethanol, then routinely processed and paraffin embedded. Five 5μm-thick serial sections were obtained, and the first, third and fifth of these were routinely H&E stained for histopathological examination. Each slide was randomly assigned a new identifier to mask exposure group from the pathologist. The first, third and fifth slides for each individual were examined for neoplasia or preneoplastic changes. Following this initial examination, one representative slide from each individual was examined and scored for histologic evidence of renal disease.

3.3.2.4. Clinical Chemistry

Measurement of serum clinical chemistry values (BUN and creatinine) was performed in house using the VetScan VS2 Chemistry Analyzer (Abaxis, Union City, CA) and the VetScan Comprehensive Diagnostic Profile Rotor. Serum samples were thawed on ice, mixed with an equal volume of 0.9% saline solution to obtain a final volume of 100 μL, pipetted into the rotor, and analyzed.

3.3.2.5. Urine Protein/Creatinine Ratio

Urine Protein Assay was performed using Pierce Coomassie Plus Bradford Assay (Thermo Fisher #23236). A urine sample aliquot was thawed on ice and the assay was performed per manufacturer protocol. Briefly, urine samples were diluted with MilliQ water (1:500 for females, 1:1000 for males) and pipetted into microplates. The Coomassie reagent was brought to room temperature before adding to all wells of the plate, and the plate was incubated at room temperature for 10 minutes. Samples were measured in duplicate using a microplate reader set at 595 nm. Urine protein concentration (mg/dL) was determined based on a standard curve.

Urine Creatinine measurement was performed using the Creatinine (urinary) Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI). A urine sample aliquot was thawed on ice and the assay was performed according to the manufacturer's protocol. Briefly, urine samples were diluted with MilliQ water and added in duplicate to the wells of a microplate. Alkaline picrate solution was added to all wells and incubated on a shaker for 10 minutes at room temperature. Initial absorbance was read at 500 nm. Acid solution was added to all wells and the plate was incubated for an additional 20 minutes at room temperature before the final absorbance measurement. Samples were measured in duplicate using a microplate reader set at 500 nm. Urine creatinine concentration (mg/dL) was determined based on a standard curve.

3.3.2.6. Urine Osmolality

Urine Osmolality was directly measured by freezing point depression using the OsmoPro Multi-Sample Micro-Osmometer (Advanced Instruments, Norwood, MA). Briefly, 20 μL of urine was pipetted into an OsmoPro tube, and 20 urine samples were analyzed at one time on the instrument. Urine osmolality was reported as mOsm/Kg H_20.

3.3.2.7. Urine Neutrophil Gelatinase Associated Lipocalin (NGAL)

Urinary Neutrophil Gelatinase Associated Lipocalin (NGAL) measurement was performed using the Mouse Lipocalin-2/NGAL Quantikine ELISA kit (R&D Systems). Urine aliquots were thawed on ice, diluted in the provided assay buffer, and the assay was performed according to the manufacturer's protocol. Briefly, assay buffer was added to all wells of the kit-provided microplate, samples and standards were added in duplicate, followed by incubation at room temperature. The plate was aspirated and washed, followed by conjugate addition and incubation, substrate incubation in light protected conditions, and addition of stop buffer. Samples were measured in duplicate using a microplate reader set at 540nm/450nm within 30 minutes of adding the stop buffer. Urine NGAL concentration was determined based on a standard curve. Urine NGAL concentrations were then normalized to urine creatinine and urine osmolality measurements to account for variability in sample concentration.

3.3.2.8. Statistical Analysis

Statistical analysis of the histological tubular disease scoring was performed to investigate the effects of TCE dose, iAs dose, and sex on renal damage. Statistical analyses were performed by Mr. Donghyuk Lee of the Texas A&M University Statistical Collaboration Center and Amie Perry. JMP 13 (JMP®, Version 13) and GraphPad Prism 8 (Windows version 8.0.1) software was used for all analyses, and figures were produced using GraphPad Prism. Data having non-normal distributions were log transformed to approximate normality before analysis. The control group for statistical analyses was defined as the No iAs/No TCE group when comparisons among groups were performed.

3.4. Results

3.4.1. Male Mice Have Greater Renal Damage

Statistical analysis of the histological tubular disease scoring was performed to investigate the effects of TCE dose, iAs dose, and sex on renal tubular damage. After determining that the data were normally distributed, ANOVA was performed ($p<0.0001$). Further analysis showed that sex ($p=0.0006$) was a significant factor, and that there was a significant interaction between TCE dose and iAs dose ($p=0.0005$). A t-test was performed, and, overall, male mice had higher mean total tubular disease scores than female mice (Figure 3.1a).

In addition, male mice in general had higher mean scores for nearly all of the assessed individual renal lesions on histologic examination, including: glomerular

amyloid/hyaline glomerulopathy ($p<0.0001$), medullary amyloid or fibrosis ($p<0.0001$), end-stage kidney ($p=0.0034$), perivascular cellular infiltrate ($p=0.005$), chronic interstitial cellular infiltrate ($p=0.028$), tubular degeneration and regeneration ($p=0.0134$), tubular single cell necrosis ($p=0.0006$), pelvic dilation ($p<0.0001$), infarcts ($p<0.0001$), and hyaline casts ($p<0.0001$).

The clinical chemistry parameters assessed had a similar pattern to the histology parameters in that male mice generally had higher concentrations of markers associated with renal damage. Among the urine and clinical chemistry parameters tested, male mice had higher urine protein creatinine ratios ($p<0.0001$), higher blood urea nitrogen (BUN) ($p<0.0001$), and higher urinary NGAL normalized against creatinine ($p=0.0027$) (Figure 3.1b-d). While urinary NGAL normalized against osmolality was numerically higher in male mice than female mice, these results did not rise to the level of significance ($p=0.1654$).

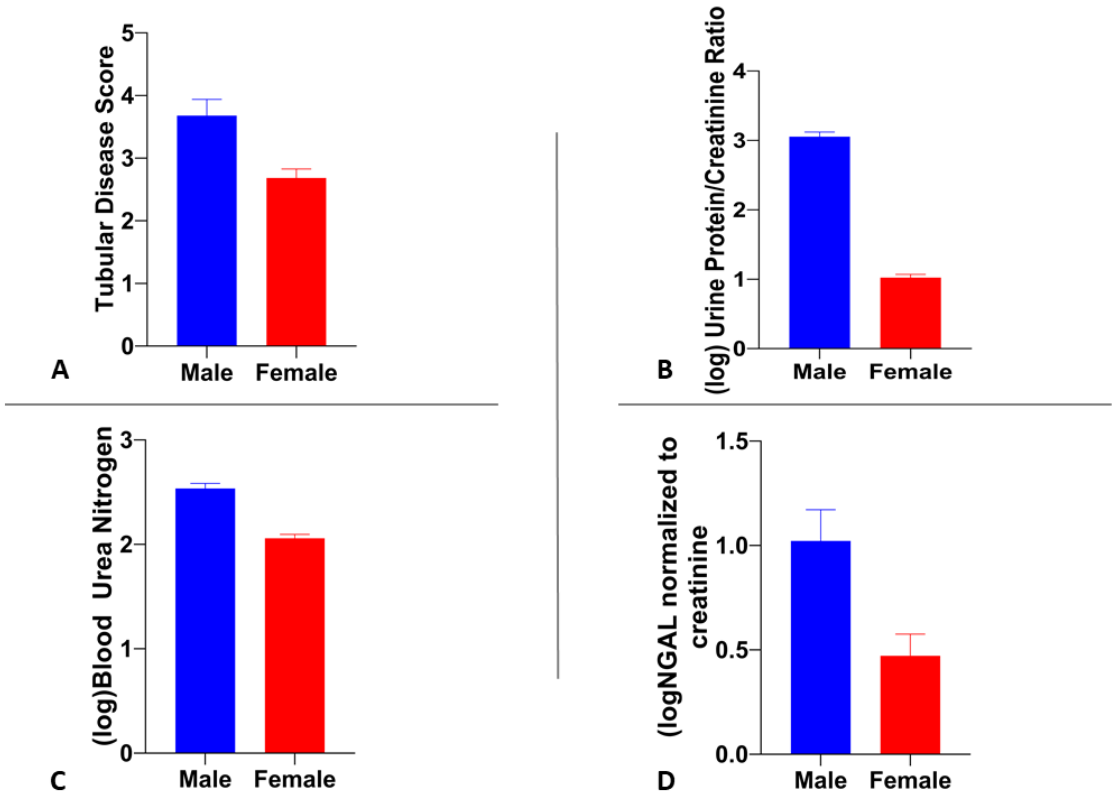

Figure 3.1 Renal Parameters for Male Versus Female Mice.
Mean (+SE) A) Tubular damage across all dose groups. Welch's t test. Mean score with standard error. Male n=171; female n=278 ($p<0.0001$). B) Urine Protein/Creatinine ratio across all dose groups. T test following log transformation. Male n = 113, female n = 233 ($p<0.0001$). Blood Urea Nitrogen across all dose groups. T test following log transformation. Male n = 156, female n = 264 ($p<0.0001$). NGAL normalized to creatinine across all dose groups. T test following log transformation. Male n = 113, female n = 233 ($p=0.0027$).

3.4.2. Inorganic arsenic does not increase histologic evidence of tubular damage in combination with low TCE

The highest damage score resulted from high arsenic and no TCE exposure, but no significant difference was observed between the high As/high TCE condition and the

high As/low TCE condition (Figure 3.2). Lowest damage occurred with no toxicants (as expected). Interestingly, when the TCE level is high no significant effects were seen with the addition of arsenic exposure. When TCE level is low, higher damage was observed without Arsenic, and lower damage scores were found when low or high arsenic was included. This finding is somewhat perplexing, as we would expect combination exposure at any level to result in more damage. It is possible that animals that had more extreme damage responses to combined toxicant exposure were lost during the exposure period of the study and thus were not available for analysis at the termination of the study. This would skew comparisons between animals with single toxicant exposure versus those that were exposed to both toxicants, resulting in the appearance of no difference between combination and single toxicant exposure or potentially even lower damage as a result of combination toxicant exposure.

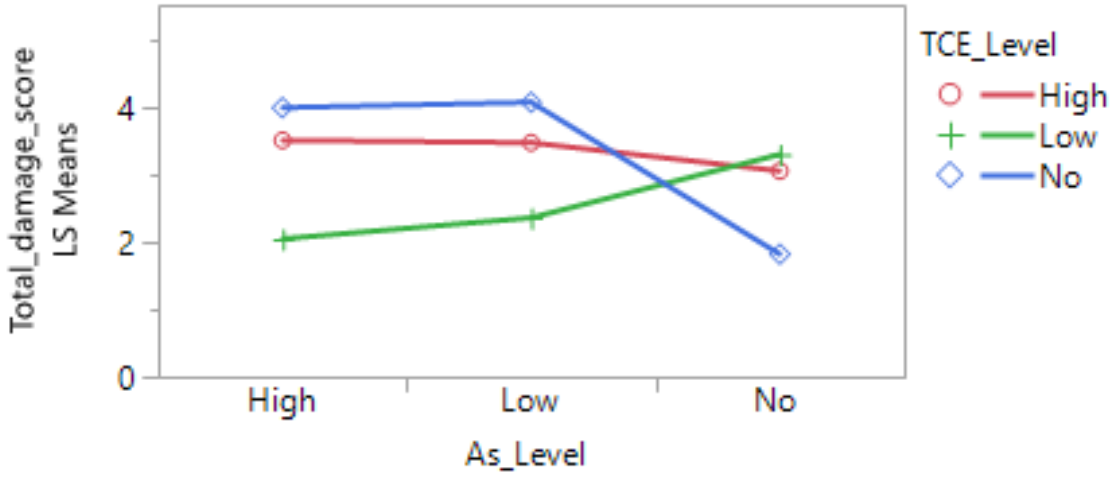

Figure 3.2 Interaction Between iAs and TCE Level.
The highest damage score was observed with high arsenic and no TCE exposure, but there was no difference between this and the high As/high TCE condition and the high As/low TCE condition. The lowest damage score was observed in the No As/No TCE group. With high or no TCE, no change in damage score was observed with arsenic exposure. When TCE level was low, higher damage was observed without arsenic, and lower damage scores were found when low or high arsenic was included.

When individual kidney lesion types were examined, those that were more common in toxicant-exposed animals (whether single or combination exposure) compared to the No As/No TCE control group were: pelvic cellular infiltrates ($p = 0.0005$), tubular degeneration and regeneration ($p < 0.0001$), tubular dilation in the OSOM ($p = 0.0022$), pelvic dilation ($p = 0.0254$), and epithelial vacuolation of tubular epithelium ($p = 0.0168$) (Figure 3.3). Among these lesions, pelvic cellular infiltrates were higher in animals exposed to each single toxicant (TCE only $p = 0.0064$; As only $p = 0.0033$) and combination-exposed animals ($p = 0.0274$) as compared to the unexposed group, but there was no difference between combination-exposed animals and single-

exposed animals. This pattern held for tubular degeneration and regeneration (TCE only mean $p < 0.0001$; As only $p < 0.0001$; combination exposure $p = 0.0011$) except that arsenic only exposure resulted in higher tubular degeneration and regeneration score compared to combination-exposed animals ($p = 0.0004$). For tubular dilation of the OSOM, both single exposure to TCE ($p = 0.0386$) and single exposure to iAs ($p = 0.0003$) resulted in higher scores for this parameter compared to those not exposed, but single exposure to iAs resulted in higher scores for this parameter compared to combination exposure ($p = 0.0100$). For tubular vacuolation, only combination exposure resulted in higher scores for this parameter and only in comparison to unexposed animals ($p = 0.0296$).

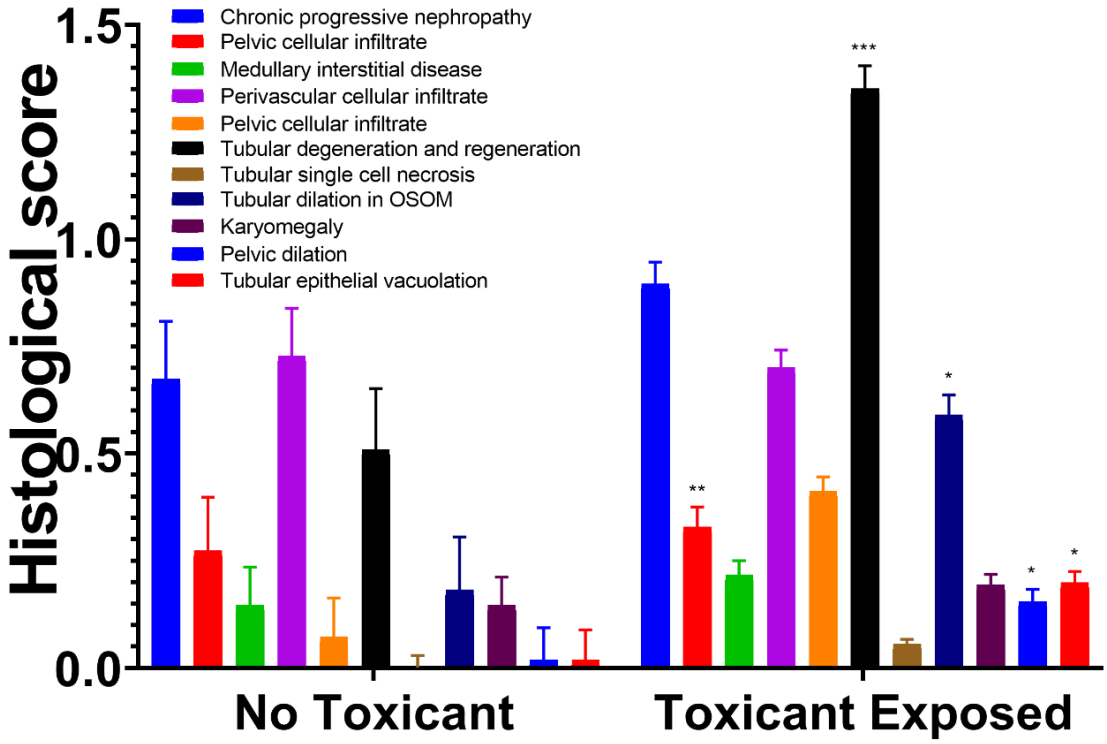

Figure 3.3 Histologic Lesions in Toxicant-Exposed vs. Non-Exposed Mice.
T-test. Mean (+SE) The individual lesion types that are more common in toxicant-exposed animals (whether single or combination exposure) compared to the No As/No TCE control group are: pelvic cellular infiltrates ($p = 0.0005$), tubular degeneration and regeneration ($p < 0.0001$), tubular dilation in the OSOM ($p = 0.0022$), pelvic dilation ($p = 0.0254$), and epithelial vacuolation of tubular epithelium ($p = 0.0168$).

3.4.3. BUN Levels – Correlation to Histology Parameters and Levels Among Groups

Blood urea nitrogen concentrations across the study population were analyzed for correlation to the tubular disease score as well as the urinalysis parameters (Table 3.1). BUN values were log transformed before Pearson's correlation testing was performed.

Moderate positive linear correlations were observed between (log)BUN and tubular score (0.4657), Kidney weight/Body weight ratio (0.4268), (log)NGAL normalized to creatinine (0.4349), and (log)NGAL normalized to osmolality (0.3687) ($p<0.0001$ for all). A slight to moderate linear positive correlation was observed between (log)BUN and UPC ratio (0.3237). The strongest correlation was found between BUN and tubular disease score.

Table 3.1 Correlation of Biomarkers with Gross and Histologic Renal Disease

	Tubular disease Score	Kidney/Body weight ratio	(log) BUN	(log) UPC ratio	(log) NGAL normalized to creatinine	(log) NGAL normalized to Osmolality
Tubular disease score	1	0.4085	0.4657	0.1700	0.3394	0.2934
Kidney/Body weight ratio	0.4085	1	0.4267	0.3693	0.3237	0.2522
(log) BUN	0.4657	0.4267	1	0.2970	0.4349	0.3687
(log) UPC ratio	0.1700	0.3693	0.2970	1	0.1498	0.0364
(log) NGAL normalized to creatinine	0.3394	0.3237	0.4349	0.1498	1	0.9724
(log) NGAL normalized to Osmolality	0.2934	0.2522	0.3687	0.0364	0.9724	1

BUN concentrations for single and combination exposed groups were compared to the No As/No TCE group after log transformation of the BUN values to improve normality. ANOVA followed by the Tukey-Kramer HSD was used for this analysis. In this analysis, BUN was higher in single iAs exposed animals compared to unexposed animals, but no other differenced were observed. BUN levels for each exposure group were analyzed and compared to the No As/No TCE group. Log transformation of BUN values was performed to approximate normality before performing ANOVA with post hoc testing using Dunnett's method. Significance was reached only in the Low As/No TCE group ($p=0.0137$) and the High As/No TCE group ($p=0.0333$) when compared to the No As/No TCE group used as the control for this analysis (Figure 3.4).

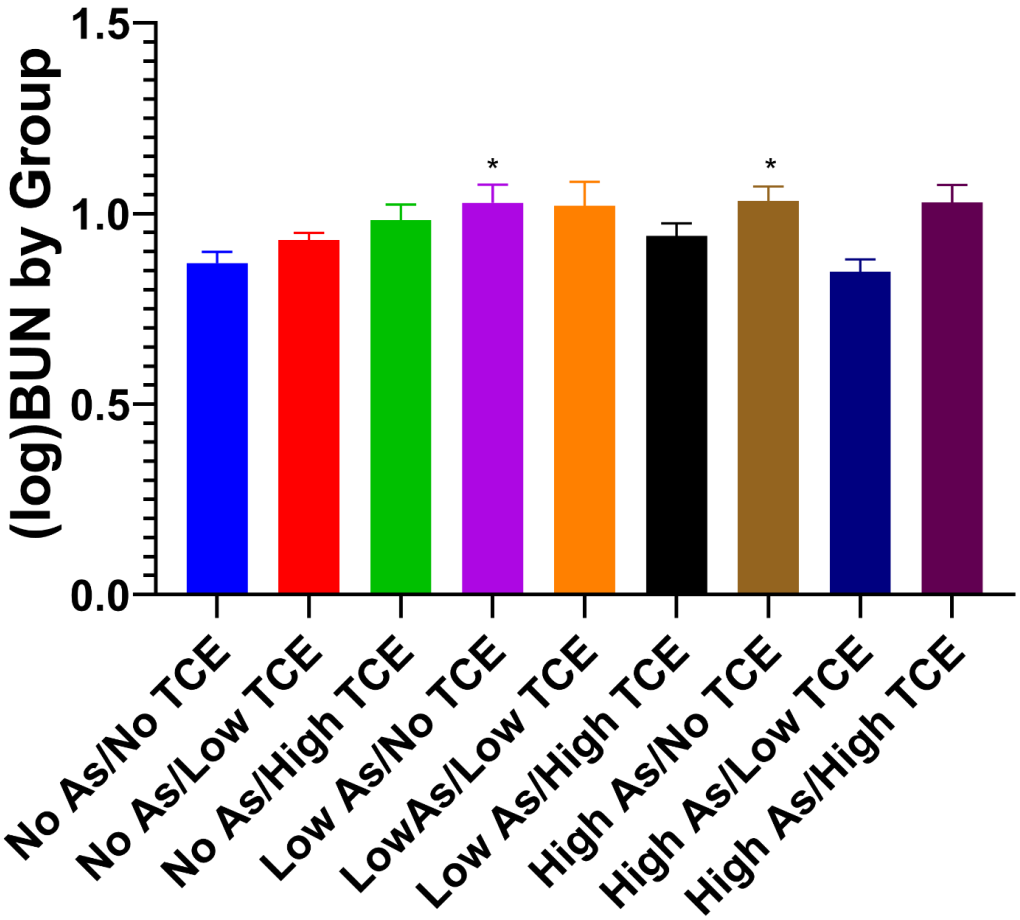

Figure 3.4 BUN Level by Toxicant Exposure Group.
Mean (+SE) log transformation of the BUN data was performed to attain normality, and then ANOVA with post hoc Dunnett's test for multiple comparisons was performed. The No As/No TCE group was used as a control for comparisons. Low As/No TCE (p=0.0333) and High As/No TCE (p=0.0137)) have higher BUN levels than the control group.

3.4.4. Urinary NGAL

The concentration of NGAL in urine was measured. Concentrations of analytes in urine may vary depending on the concentration of the urine as well as renal handling

of a given analyte, and urine concentration may vary greatly depending upon hydration and kidney function. For this reason, urine analytes are normalized against a substance that should be relatively constantly excreted, regardless of the concentration of the urine. Usually this is done using creatinine because it is easy to measure, and, assuming constant production of creatinine and stable glomerular filtration rate, its excretion should be constant as it is not reabsorbed or secreted by the renal tubules. The gold standard would be to normalize against osmolality because it is a measure of the molecules in the urine rather than a surrogate for that measure, such as creatinine. However, it is rarely done because of its expense and the relative ease of measuring creatinine.

In the present study, both urine creatinine and urine osmolality were measured and used to normalize urine NGAL, and results using the two methods were compared. Strong linear correlation (0.9724) was observed between NGAL normalized against creatinine and NGAL normalized against osmolality after log transformation (Table 3.1).

Despite its reported association with tubular damage and slight to moderate correlation with the tubular disease score obtained from histologic examination of the kidneys (0.3395 for NGAL normalized against creatinine and 0.2934 for NGAL normalized against osmolality), no difference was observed in urine NGAL normalized against creatinine ($p=0.4443$) (Figure 3.5) or NGAL normalized against osmolality ($p=0.4746$) (not shown) among the exposure groups, or between single or combination exposed animals compared to the No As/No TCE group after log transformation of the data.

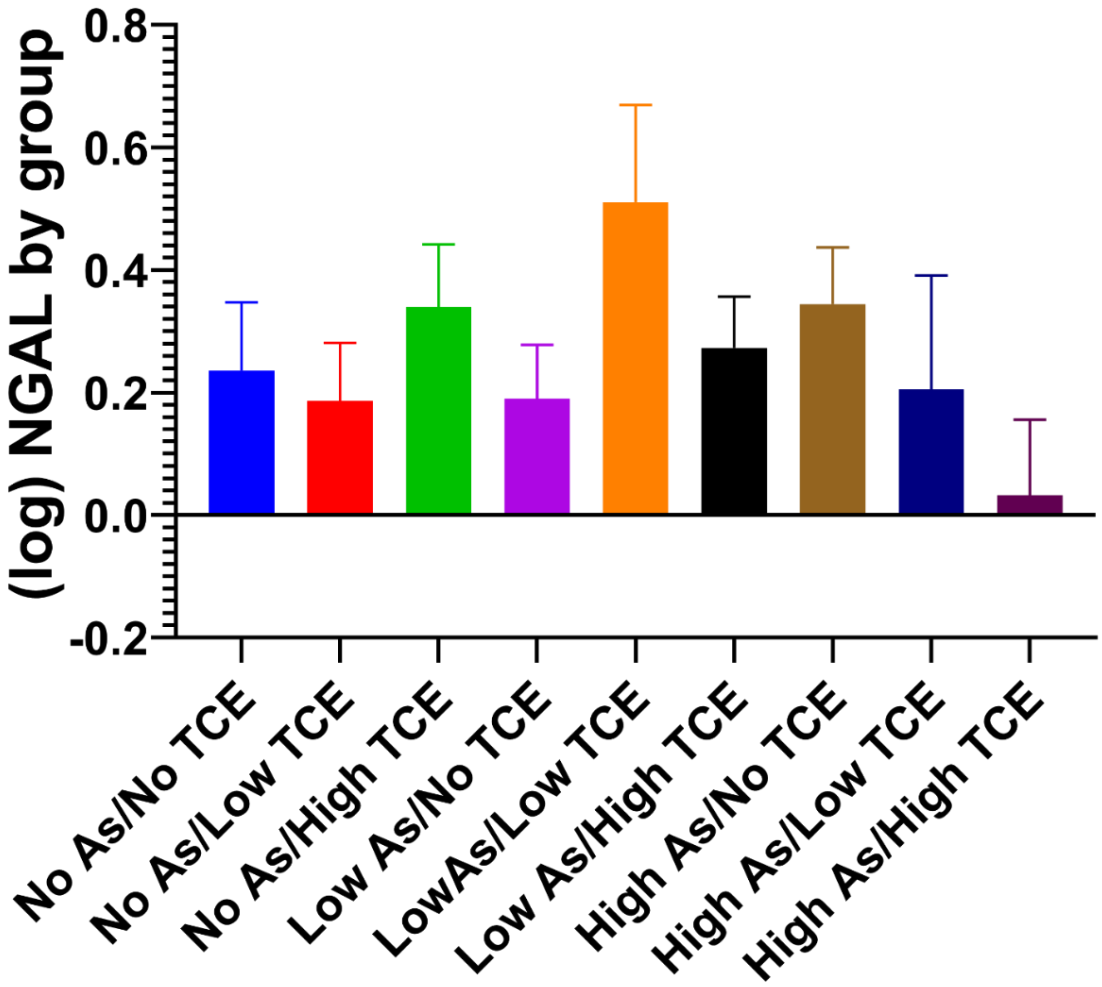

Figure 3.5 Urinary NGAL by Toxicant Exposure Group.
Mean (+SE) ANOVA with No As/No TCE group used as control. Urinary NGAL normalized against urinary creatinine. No significant difference from the No As/No TCE group was observed in any of the toxicant-exposure groups.

3.4.5. Urine Protein/Creatinine Ratio

Urine protein creatinine ratio is used to evaluate the level of protein in the urine (proteinuria). When UPC ratios are compared between the toxicant-exposure groups and the No As/No TCE group used as a control, the UPC ratio is higher only in the Low As/High TCE group ($p=0.0082$) and the High As/No TCE group ($p=0.0145$) (Figure 3.6). Male mice across the entire study population had higher UPC values ($p<0.0001$) (Figure 3.7A), which was expected given that male mice are known to have higher levels of urinary proteins than female mice physiologically. When UPC levels for the entire study population were compared to the UPC levels in the no toxicant control group, the magnitude of the increase was higher among toxicant-exposed mice than in the control population (Figure 3.7B).

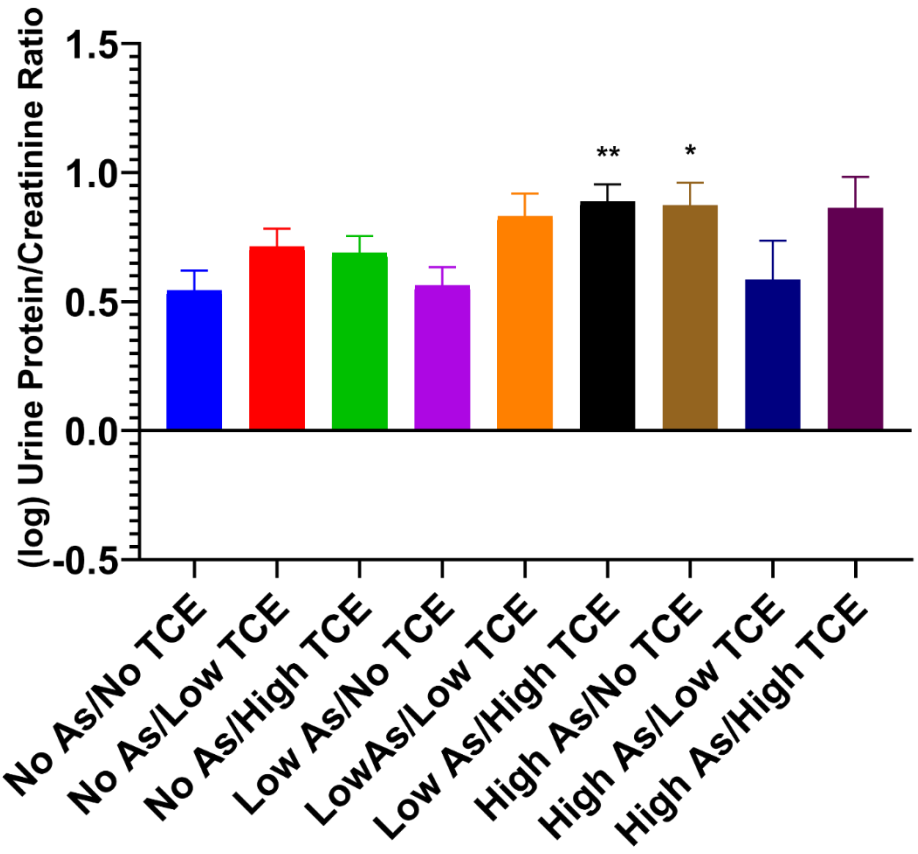

Figure 3.6 Urine Protein Creatinine Ratio by Toxicant Exposure Group. Mean (+SE) ANOVA with No As/No TCE group used as control. UPC ratio is higher only in the Low As/High TCE group (p=0.0082) and the High As/No TCE group (p=0.0145).

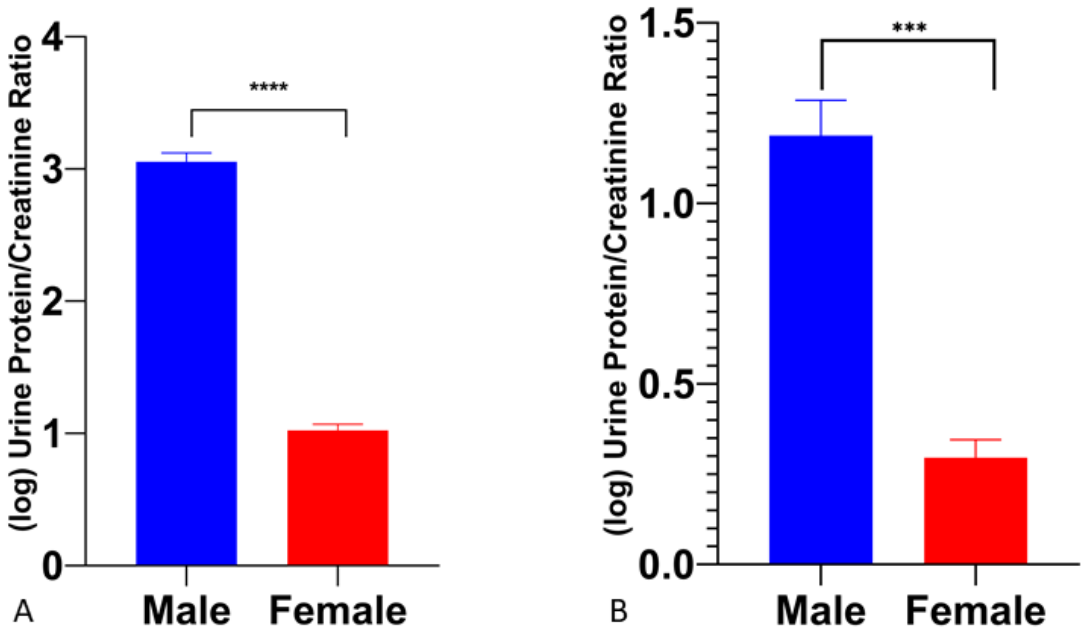

Figure 3.7 UPC Ratio in Male vs. Female Mice
Mean (+ SE) Welch's t-test. UPC ratios for male versus female mice across the study population. Male mice had significantly higher UPC ratio than female mice both across the entire study population (A), and in the no toxicant exposure group (B) but the magnitude of the difference was greater in toxicant exposed animals.

3.5. Discussion

As discussed in Chapter 2, the development of a rodent model of tumor development caused by human exposure to TCE has been challenging. Traditional toxicological studies evaluate the effects of toxicants in genetically homogeneous populations and in isolation, which does not reflect the human population or exposure situation. Another challenge is determining how combination exposure to environmental toxicants may affect renal health.

The kidney is responsible for many essential bodily functions, and it is particularly important in metabolism and excretion of drugs and environmental toxicants (Perazella, 2009). Because it is involved in metabolism and excretion of many potentially toxic agents, it is particularly vulnerable to injury from these agents. Many factors beyond toxicant exposure may affect renal disease, and this, along with the relatively late detection of disease in humans, makes it challenging to study. Chronic kidney disease has been linked to chronic environmental toxicant exposure (Kataria, et al., 2015; Soderland, et al., 2010). Given the likelihood of combination exposure and other factors that may affect susceptibility to the development of CKD, it is important that the effects of combination toxicant exposure are studied. In this study we analyzed the renal effects of long-term combination exposure to TCE with or without iAs.

Detection of kidney disease in early stages can be difficult. The gold standard of kidney function analysis is measurement of glomerular filtration rate (GFR). In the past, GFR measurement in mice was a terminal procedure and required multiple blood collections. More recently, techniques for measurement of GFR have been developed that are not terminal, but these techniques still require multiple blood collections (Rieg, 2013), reducing the blood volumes available for other analyses and rendering these methods impractical for our purposes. Therefore, serum and urinary biomarkers are more commonly used to assess kidney function.

While the use surrogate markers of GFR (BUN and creatinine) is well established, they have relatively poor sensitivity and specificity for renal toxicity, and they may be affected by non-renal factors such as muscle mass and dehydration.

Elevation of urinary NGAL has been reported with tubular injury. It is considered a good biomarker for acute kidney injury and is considered promising as a biomarker in CKD (Wasung *et al.*, 2015). Because blood volumes available from mice are small, we evaluated the UPC and urinary NGAL as more sensitive indicators of kidney damage, alongside histopathologic examination of the kidney and the classical biomarker BUN.

In this study, consistent with previous histology results, some renal biomarkers show evidence of renal health effects in some treatment groups, there is no evidence of increased renal damage with combination exposure to these toxicants as opposed to single exposure to one toxicant. As reported, lower renal biomarker concentrations were observed with low TCE and any level of arsenic than with TCE alone and with high TCE no increase in damage is observed with arsenic exposure. This finding agrees with the unexpected finding from Chapter 2 that TCE and iAs coexposure, regardless of the toxicant level involved, did not result in increased histological evidence of renal damage. As in that case, we speculate that the degree of damage caused by the first toxicant may have caused significant enough damage to cause death of the cells and require a regenerative response, precluding additional damage accumulation from the second toxicant.

When histologic parameters were examined, it was found that male mice in general had higher levels of tubular damage on the composite tubular disease score. They also had higher scores for many of the individual histologic lesions, including those not included in the composite tubular disease score, such as glomerular disease. This is expected, as male rodents generally have a higher incidence of renal disease,

including chronic progressive nephropathy. The reason for this increase in renal disease in male rodents is not entirely understood but is well documented. In addition to the histologic evidence of renal disease, male mice also had higher UPC ratios, BUN, and urinary NGAL levels than female mice, supporting the histological findings. Male mice generally do have higher UPC values than females because of higher levels of protein in the urine of male mice, primarily the major urinary proteins (MUPS).

Several individual renal lesions scored on histologic examination of the kidneys were increased in animals exposed to either or both toxicants when compared to those in the No As/No TCE group. These include pelvic cellular infiltrates (a measure of inflammation), as well as tubular degeneration and regeneration, tubular dilation in the outer stripe of the outer medulla, and tubular epithelial vacuolation. This is consistent with previous findings of higher overall tubular disease score in toxicant exposed groups. Among these individual lesions, only tubular epithelial vacuolation was higher in animals exposed to both toxicants than in unexposed animals, although no difference was observed for this parameter between the combination exposed groups and those exposed to only TCE or only iAs.

Evaluation of urinary NGAL, UPC, and BUN did not show a strong pattern in response to toxicant exposure. Urinary NGAL levels were not different in single or combination exposed animals across the study, nor in any single exposure group when compared to the No As/No TCE group. However, urinary NGAL was higher in male mice than in female, consistent with the histologic finding of generally higher evidence of renal damage in male mice than in females. Similarly, the UPC ratio was higher in

male mice than in female mice, as expected given the generally higher levels of urinary proteins in male mice. But, the UPC ratio was only elevated above that in the No As/No TCE group in the Low As/High TCE group and the High As/No TCE group. The common factor between these groups is As exposure. BUN was higher in male mice as well, consistent with the overall pattern seen in male mice, but it was also higher in the Low As/No TCE and High As/No TCE group. Like with UPC ratio, the common factor was As exposure, and TCE exposure appears to have had little effect on the BUN concentration.

Correlation between the measured parameters was examined. As described, the highest correlation, although still only moderate, was observed between the histologic tubular disease score and BUN. Similar correlation was observed between BUN and the gross measurement of kidney weight/body weight ratio. Tubular disease score and BUN also had slight to moderate correlations with urinary NGAL, even though urinary NGAL did not reach significance in association with any specific toxicant exposure profile. NGAL normalized against creatinine and NGAL normalized against osmolality had strong positive correlation, indicating that normalizing against creatinine is likely an acceptable normalization method in this study.

As detailed in Chapter 2, there are significant limitations to this study including significant loss of animals before the end of the study (44% of the study population), limiting the number of samples available for analysis. It is possible that some of this loss was due to extrarenal effects of TCE and iAs, as both are known to have effects on other organs that were not the focus of this study. Also possible are losses from conspecific

aggression, a known issue with male mice in particular. As described in Section 2, efforts were made to limit conspecific aggression that has the potential to cause losses.

Another limitation is that there is very little data on expected age-related or background lesions in the specific strains of mice used as the parental background strains in the present study, and there is no information regarding crosses derived from these strains.

Potentially, the decision to deliver iAs in food instead of the usual vehicle, drinking water may be a limitation. This was done to mimic ingestion of low doses of iAs in food, but might alter the absorption, distribution, metabolism, and excretion characteristics of iAs. In addition, we wished to use food for one toxicant and water for delivery of the other. Because iAs is better documented as a contaminant of food than TCE, we decided to use food as the vehicle for iAs and water for TCE.

In addition, other potential biomarkers of renal disease could have been chosen, including one of the classical markers, serum creatinine, and one of the newer biomarkers, KIM-1. Due to the limited volume of blood available, it was difficult to obtain the volumes of serum necessary to measure creatinine as well as other serum analytes. The test panel used for serum clinical chemistry was not sensitive enough to detect creatinine levels in the volume of sample available. For this reason, we chose to use BUN as our classical serum marker of renal disease. In many cases, urine volumes were limited as well. While multiplex assays for urinary biomarkers are available, they were considered cost-prohibitive for a study this large, and one urinary biomarker was chosen to accompany measurements of urinary creatinine, osmolality, and protein.

Because elevated NGAL has been associated with both acute renal injury and chronic kidney disease, we chose this biomarker.

Despite the limitations of the study, this model more accurately reflects human exposure conditions by including multiple environmental toxicants, genetic heterogeneity, and long exposure to the toxicants. Despite this more accurate modeling of toxicant exposure, no evidence of increased damage from combination exposure was observed. Instead, there was evidence of increased damage due to iAs exposure, and no increase in damage with combination exposure. This may be due to damage from the second toxicant being limited by damage from the first being severe enough to necessitate regenerative responses in the renal tubular epithelium, such that a threshold of damage was reached. It is possible that the toxicants had antagonistic effects, although more analysis would be required to confirm this. More studies of combined toxicant exposure in genetically heterogeneous populations are needed, and it is likely that the effects of different toxicants in combination will have differing outcomes.

4. CONCLUSIONS

4.1. Modeling of Biological Response to Toxicant Exposure

TCE and iAs are environmental toxicants that target the kidney as well as other organs. Chronic exposure to these toxicants has been associated with various renal health effects including development of clear cell renal cell carcinoma in humans with chronic exposure to TCE and chronic kidney disease with exposure to either of these toxicants. Specific responses to toxicant exposure is influenced by many factors other than just the toxicant itself. These factors include intrinsic (genetic and epigenetic variation, age and life stage, sex) and extrinsic factors (co-exposures to other toxicants, nutritional state, stressors, dosage, co-morbidities) (Zeise, et al., 2013).

Development of a rodent model for renal cancer and disease development due to toxicant exposure is complicated by differences in renal handling of toxicants between rodents and humans as well as the tendency of rodents to develop significant background spontaneous renal disease that may mimic pre-neoplastic disease or mask more subtle lesions. In addition, classical toxicological studies often focus on one potential toxicant in a genetically homogeneous population, in contrast to the more typical exposure situation that involves genetically heterogeneous populations and exposure to mixtures of toxicants.

Given these challenges, it has become clear that toxicological studies must address the effects of genetic variability and the range of sensitivity to toxicity due to this inherent variability. Experimental paradigms that assess and control for as many of

the intrinsic and extrinsic factors influencing renal response to toxicant exposure are also needed. Human epidemiological studies often lack detail regarding exposure levels, potential for co-exposure to toxicants, chronicity of exposure, or other potential risk factors including smoking or obesity. In an effort to address these limitations, a mouse model was devised that included genetic heterogeneity, mixtures of toxicants at environmentally relevant concentrations, and a diet that reflects a typical western diet to better reflect the exposure conditions of human populations.

Primary renal cell tumors were not observed in this study. It is possible that there were too few mice in the study to detect this effect, given the rarity of the tumors even in exposed human populations. Despite the lack of primary renal tumor development in this study, evidence of differences in renal disease were observed among the different treatment groups.

Increases in tubular disease scores between animals exposed to TCE alone, exposed to iAs alone, and those exposed to both toxicants were observed, although no difference in severity was observed in co-exposed animals, contrary to our expectations. The strongest pattern in the parameters evaluated was that male mice had higher evidence of renal disease than female mice. Both of these patterns largely held across histologic examination. Individual lesions that were increased in toxicant-exposed animals compared to unexposed included pelvic cellular infiltrates, tubular degeneration and regeneration, tubular dilation in the outer stripe of the outer medulla, and tubular epithelial vacuolation. The only individual lesion score that was higher in animals exposed to both toxicants than in unexposed animals was tubular epithelial vacuolation,

although, similar to the tubular disease score results, no difference was observed for this between combination exposure and single toxicant exposure.

As detailed in Section 3, evaluation of BUN, urinary NGAL, and UPC showed no strong pattern in response to toxicant exposure except that, like in the histological parameters, male mice had overall higher levels than female mice. Urinary NGAL, serum BUN, and UPC ratio were all higher in male mice than in female mice when all mice were considered.

In contrast, urinary NGAL levels were not different in single or combination exposed animals across the study. Increased BUN was observed in animals exposed to As alone when single or co-exposure was examined. Among the individual treatment groups, BUN was higher in the Low As/No TCE and High As/No TCE group. UPC ratio was higher in the Low As/High TCE group and the High As/No TCE group. The common factor among these parameters was As exposure. Exposure to TCE appears to have had little effect on the biomarkers. In some cases, there were very few samples from male mice to evaluate, and so it is possible that there simply was not enough power to detect effects for some of these measurements, and this may have affected our ability to detect significant differences with these parameters even had there been a difference.

It is possible that damage caused by one toxicant was not increased by exposure to a second renal toxicant as the cells already damaged by the first toxicant to the point of requiring regeneration would not be further damaged by the second toxicant.

Moderate correlation was observed between several histologic parameters, BUN, and urinary NGAL despite the lack of significant differences between treatment groups

with urinary NGAL. The tubular disease score and BUN had the highest correlation among the measured parameters and a similar degree of positive correlation was observed between BUN and kidney weight/body weight ratio. It is speculated that the correlation between these measurements may be related to the significant increase in BUN, kidney weight/body weight ratio, urinary NGAL, and UPC in male mice across the study, rather than related to the toxicant exposure.

Despite efforts to increase the carcinogenic potential of the renal toxicants in the study by including two toxicants whose primary target is the kidney at varying doses over a long period of time, no primary renal tubular neoplasms developed. Given the results observed, it appears that iAs is the greater contributor to renal damage in our study, and this is possibly because efforts were made to alter the renal handling of heavy metals by knocking out the MDR1 gene in these mice. As previously stated, this has been shown to increase renal accumulation of heavy metals in mice. On the other hand, the renal handling of TCE was not modified, partially because its handling is complex and not fully elucidated, so it is difficult to pick a single target. Instead, the inclusion of iAs as a second toxicant was intended to potentially create an additive or synergistic effect that would increase the carcinogenicity and renal damage effects of TCE. In the present study, this effect was not observed.

Despite the limitations of the study, discussed in detail in previous Sections, this model more accurately models human exposure conditions. Unfortunately, even with this more accurate modeling, no renal cancers developed, and evidence of increased renal damage in response to co-exposure to toxicants was not observed. There was some

evidence of reduced or equal damage from co-exposure, and we speculate that this is due to a threshold effect of damage from a first toxicant limiting the ability of a second toxicant to cause damage or early loss of animals that might have had increased damage, limiting our ability to detect increased damage. Additional studies of combined toxicant exposure are needed, and since the inception of the present study, new genetic mouse resources have been developed that may help in future studies.

4.2. Future Work

The development of the genetically heterogeneous mice used in this project was time, cost, and labor intensive, and generated a large number of mice that were not used for the study protocol. While this method of generating mice for the present study was appropriate at the time, newer, less labor- and time-intensive mouse model resources are now available. A recent review article (Harrill *et al.*, 2017) has provided an overview of the various mouse resources currently available. Briefly, the main mouse populations available are the classic inbred strains, conventional outbred stocks, Mouse Diversity Panel (MDP), Collaborative Cross (CC), and Diversity Outbred (DO) models.

At the time that the present study was begun, the Collaborative Cross (CC) panel of mice was still in development, (Churchill *et al.*, 2004) and although development of additional CC strains continues, there are now a number of CC strains available that enable study of complex traits on a genetically heterogeneous but well-characterized mouse model. Indeed, recently, the first toxicological study utilizing CC mice was published (Cichocki, et al., 2017), indicating that there is a large amount of variability

among the CC strains in metabolism and response to tetrachloroethylene, a molecule related to TCE. This result strengthens the argument for use of genetically heterogeneous, but defined mouse models such as the CC mice to predict the variability in human sensitivity to toxicants.

Future development of a mouse model of human toxicant-induced clear cell renal cell carcinoma may utilize one of the newer mouse model resources designed to help study the genetic influences on multifactorial diseases. In addition, further elucidation of the differences in metabolism of TCE in mice versus humans, and the genes involved in this process, may provide avenues for manipulation of the mouse to more accurately reflect the metabolic process as it exists in humans and the toxic metabolite profile that results in the development of renal tumors due to TCE exposure.

One possibility for further genetic study using the present model and existing data is the use of quantitative trait locus (QTL) analysis on the animals that had exaggerated responses compared to the animals that had muted responses to toxicant exposure to elucidate potential genes involved in this response. Examining the data from BUN, histologic scoring, and urinary NGAL, it is observed that in each category there is a subset of individuals with evidence of relatively extreme values for these analytes, indicating renal disease (Figures 4.1, 4.2, and 4.3a and 4.3b).

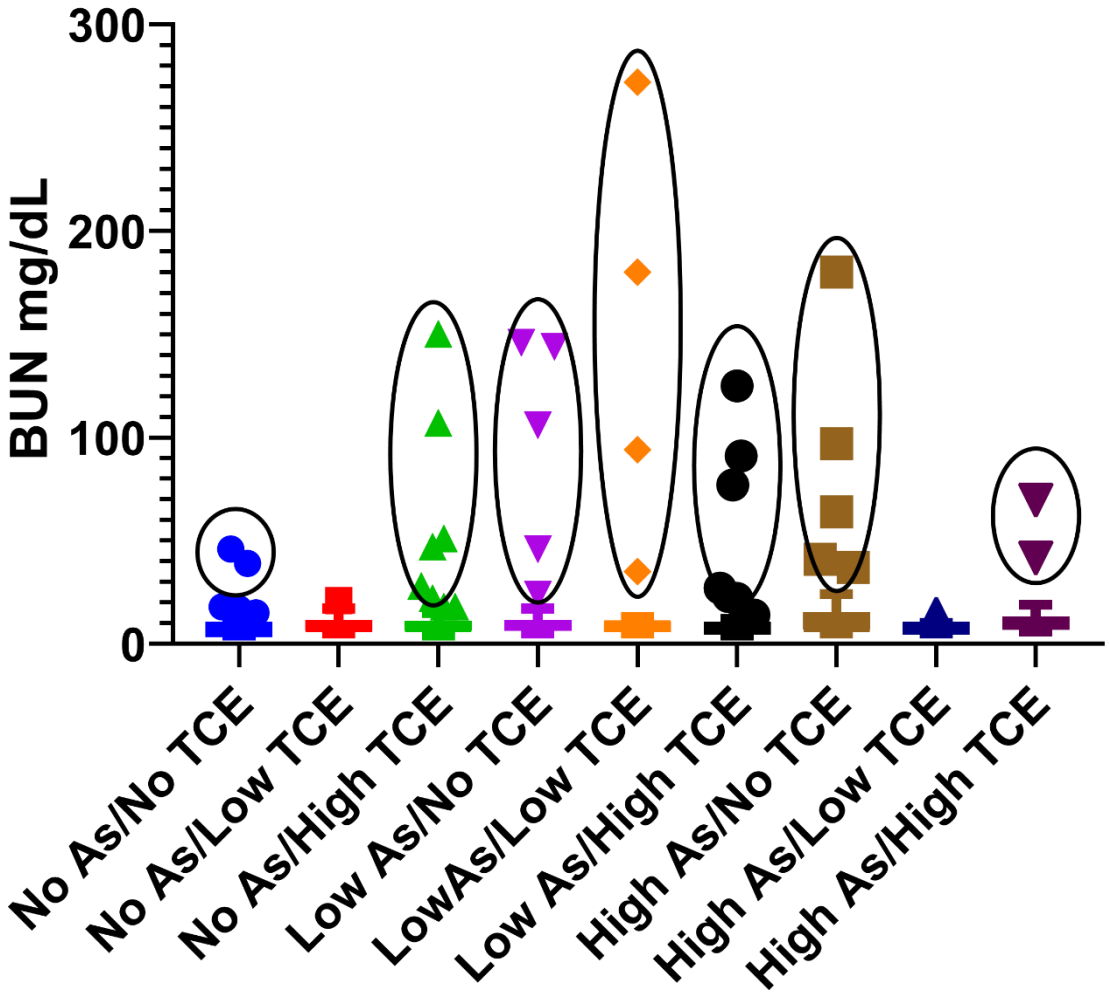

Figure 4.1 BUN Extreme Responders
Outliers determined by Tukey method.

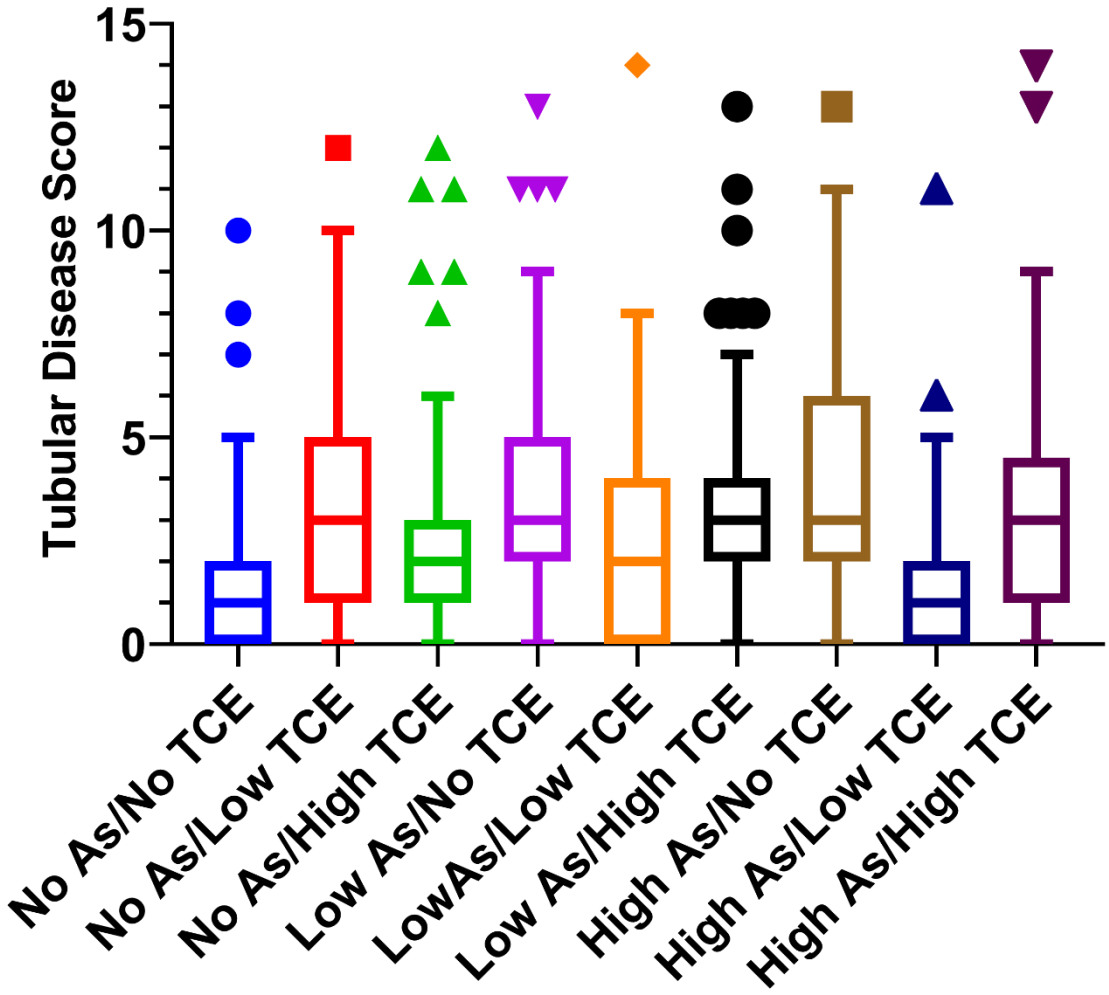

Figure 4.2 Tubular Disease Score Extreme Responders
Outliers determined by Tukey method. Median and interquartile range.

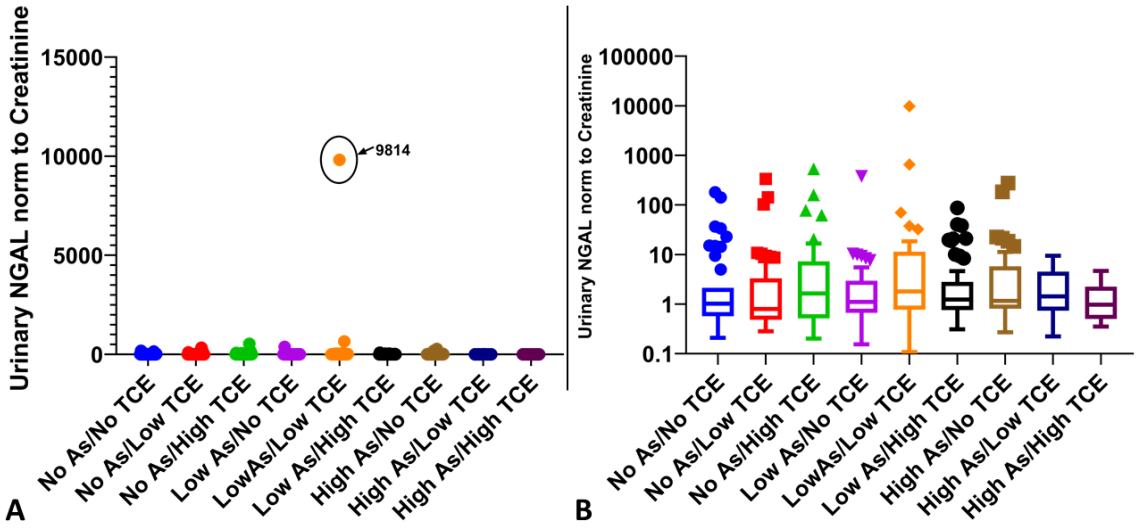

Figure 4.3 Urinary NGAL Normalized to Creatinine Extreme Responders. Outliers determined by Tukey method. A) Actual values plotted. One value skews the graph. B) Values plotted on a log 10 scale.

When these extreme responders are compared, there is some overlap between categories (Figure 4.4). This extreme response may be used as a phenotype (trait) for QTL analysis. Single nucleotide polymorphism (SNP) sequencing has been performed and QTL analyses will be performed in future studies to examine the potential for genetic influence on these differences in response.

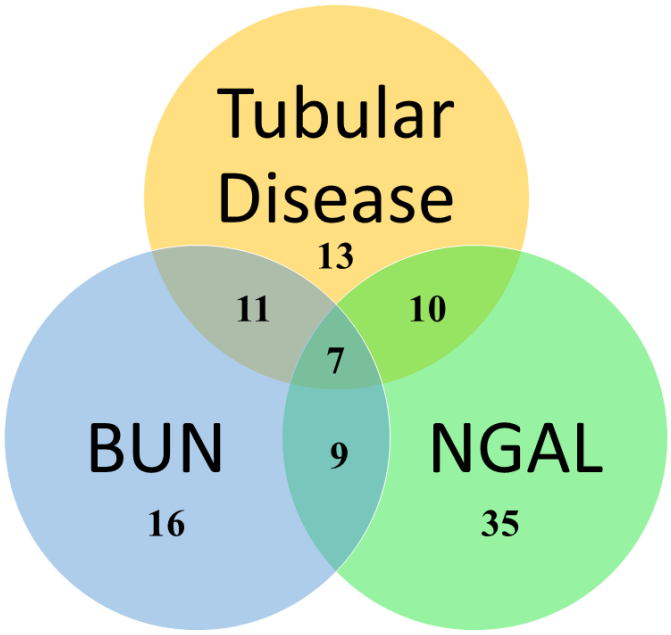

Figure 4.4 Overlap in Extreme Responders for Tubular Disease, BUN, and NGAL. Overlap among extreme responders in the three categories.

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