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Review: Risk Assessment Implications of Variation in Susceptibility to Perchloroethylene Due to Genetic Diversity, Ethnicity, Age, Gender, Diet and Pharmaceuticals

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ABSTRACT

Current cancer risk assessments do not adequately consider impacts of human inter-individual variability on susceptibility to environmental pollutants like perchloroethylene (PCE). PCE is metabolized through both oxidative and glutathione (GSH) conjugation pathways. Toxicity criteria derived using both pathways are 23-fold more stringent than those calculated using only oxidative metabolism. While toxicokinetic modeling of PCE metabolism predicted very high variability through the GSH conjugation pathway, it is unclear if the range in estimates is due to human variability or uncertainty. Thus, the variation in the GSH conjugation pathway of PCE metabolism due to genetics, ethnicity, age, gender, diet and pharmaceutical co-exposures is examined. Genetic polymorphisms were found at several loci including, *GSTT1*, *GSTM1*, *CCBL1*, *AGXT2*, *NAT8*, *ACY3*, *MRP2*, *OAT1/3*, *FMO3* and *CYP3A* that code for enzymes /transporters in the GSH conjugation pathway. Genetic diversity in *GSTT1*, *GSTM1*, and *CCBL1* between ethnic populations, as well as age, gender, diet and pharmaceutical co-exposures influences toxic and mutagenic metabolites produced through this pathway. Given this diversity,

large differences in PCE metabolism through the GSH conjugation pathway are expected. To be health protective for diverse ethnic populations and lifestyles, both the oxidative and GSH conjugation pathways need to be considered in developing PCE toxicity criteria.

Keywords

Perchloroethylene; genetic variation; susceptibility; risk assessment; glutathione

INTRODUCTION

It is difficult to quantify and incorporate the known genetic differences within the human population when developing toxicity criteria used in human health risk assessments. For example, the population of the United States and the State of California are highly diversified. According to the Henry J. Kaiser Family Foundation, the population of California in 2015 consisted of 39% White, 38% Hispanic, 15% Asian, 6% Black, 2% two or more races, and 1% Pacific Islander (Kaiser Family Foundation 2017). In addition to genetics, other factors including age, gender, diet and pharmaceutical co-exposures can also influence sensitivity and susceptibility to environmental toxicants. As stated in Chiu et al (2014) "one of the biggest gaps in risk assessment, as identified by the NRC (2009), is that inter-individual variability is not being addressed at all (in animals), or incompletely (in epidemiological studies). There is a crucial need for the development of approaches to estimate the quantitative impact of human inter individual variability in personal risk from chemical exposures (Zeise et al. 2013)…."

Perchloroethylene/Tetrachloroethylene (PCE) is a solvent widely used in the dry cleaning industry and as a degreasing agent in automotive and metal cleaning industries. A total of 458 million pounds of PCE was produced by the three major manufactures in the United States in 2011 (ATSDR 2015). PCE contamination is found in air, water, and soil (IARC 2014). The US Environmental Protection Agency (US EPA) has identified PCE contamination in at least 945 current or former National Priorities List (NPL) sites (ATSDR 2015). The California EPA, Department of Toxic Substances Control has identified PCE contamination in over 650 sites.

Metabolism of PCE proceeds both through an oxidative pathway mediated by cytochrome P450s (CYP) enzymes and a glutathione conjugation (GSH) pathway. To derive the inhalation toxicity criteria for use in human health risk assessments, a harmonized physiologically-based pharmacokinetic (PBPK) model of PCE toxicokinetic metabolism in mice, rats and humans (Chiu and Ginsberg 2011) is used by several regulatory agencies. The model fitted oxidative metabolism with about a 2-fold residual uncertainty but predicted that the estimated rates of GSH conjugation of PCE varied about 3000-fold in humans and 60-fold in mice (USEPA 2012). Inclusion of the glutathione conjugation pathway and oxidative pathway leads to a 23-fold more stringent inhalation toxicity criteria for PCE than if only the oxidative pathway is considered. Three State Regulatory Agencies recommend using PCE inhalation toxicity criteria derived from modeling PCE toxicokinetic metabolism through the glutathione conjugation and oxidative pathways (MassDEP 2014; MDH 2014; OEHHA 2016a). In contrast, the US Federal Regulatory Agency considered that it is unclear if the range in estimates in the glutathione conjugation pathway is due to human variability or uncertainty and recommends using PCE inhalation toxicity criteria derived only from modeling PCE toxicokinetic metabolism through the oxidative pathway (USEPA 2012).

Genetic polymorphisms in the GSH conjugation/β-lyase pathway have been shown to result in enzyme deficiencies, altered enzyme activities, altered physiological/pharmacological function and/or risk of cancer and disease (Bruning et al. 1997; Buzio et al. 2003; Juhanson et al. 2008; Moore et al. 2010; Suhre et al. 2011; Riedmaier et al. 2012; Yu et al. 2014). Diverse strains of mice differ in toxic metabolites produced by oxidative metabolism and by the GSH conjugation/β-lyase pathway of PCE and trichloroethylene (TCE) metabolism (Bradford et al.

2011; Chiu et al. 2014; Cichocki et al. In Press). The GSH-conjugation/β-lyase pathway can produce several reactive metabolites that are highly toxic and mutagenic. The data suggest that a high degree of human ethnic/individual variability is expected in several enzymatic steps in the GSH conjugation/β-lyase pathway of PCE metabolism due to ethnic diversity, genetic polymorphisms in pathway enzymes, as well as, variability due to age, gender, diverse diets, and pharmacological co-exposures that influence enzymatic activity (Bruning et al. 1997; Mugford and Kedderis 1998; Landi 2000; Steinkellner et al. 2001; Cabrera-Abreu and Green 2002; Lash et al. 2007; Vallon et al. 2008; Moore et al. 2010; Polimanti et al. 2013; Lash et al. 2014; Rouhou et al. 2015).

To shed light on the variability of the GSH conjugation pathway in humans, a biochemical and molecular genetic approach is used to examine the genetic and ethnic variation in the genes/enzymes controlling the pathway.

REVIEW

Carcinogenicity of PCE

The National Toxicology Program (NTP) 14th Report on Carcinogens, The International Agency for Research on Cancer (IARC), and US EPA classify PCE as reasonability anticipated to be a carcinogen (NTP 2016), probably carcinogenic (IARC 2014) or likely to be carcinogenic by all routes of exposure (USEPA 2012) to humans, respectively. In 1988, PCE was listed on California's Proposition 65 list as a chemical "known to the state to cause cancer" (OEHHA 2016b).

Metabolism of PCE

PCE metabolism has been reviewed by Lash and Parker (2001), Guyton et al. (2014), IARC (2014) and (Cichocki,Guyton, et al. 2016). For the oxidative pathway (Figure 1), PCE is oxidized by cytochrome P450s including CYP2B1/2 and CYP3A1 (Costa and Ivanetich 1980; IARC 2014) and CYP2E1 (Lash and Parker 2001; Lash et al. 2007) to produce a number of metabolites including trichloroacetyl chloride (TCAC), which then hydrolyses to yield the toxic metabolite, trichloroacetate (TCA). While CYP2E1 has an important role in the metabolism of TCE, the importance of this enzyme in the metabolism of PCE is uncertain (Lash and Parker 2001; Hissink et al. 2002; Lash et al. 2007; Lash et al. 2014). TCE is a solvent used to clean metal parts, to make other chemicals, including the refrigerant, HFC-134a, and is a degradation product in the dechlorination of PCE (ATSDR 2014).

PCE conjugation with GSH (Figure 2) forms trichlorovinyl glutathione (TCVG), which is then further processed by γ-glutamyl transpeptidase (GGT) and cysteinylglycine dipeptidase (DP) in specific tissues, and primarily the kidneys, to form the cysteine conjugate *S*-trichlorovinyl-l-cysteine (TCVC). TCVC is a critical branch point in the metabolism of PCE. TCVC may be bioactivated by β-lyase (CCBL) and/or by alanine-glyoxylate amino transferase (AGAT/*AGXT2*) to form the reactive trichlorovinylthiol (TCVT), which rearranges to form the highly reactive and mutagenic thioacylating species, DCTK. TCVC can also be activated by flavin-containing monooxygenases (FMO3s) or CYP3A to form the potent nephtrotoxicant, TCVC sulfoxide that covalently binds and may crosslink proteins (Ripp et al. 1997; Elfarra and Krause 2007; Irving and Elfarra 2013; IARC 2014). Alternatively, TCVC may (reversibly) undergo *N*-acetylation by

cysteine conjugate *N*-acetyltransferase (NAT8) to the non-toxic mercapturate *N*-acetyl trichlorovinyl cysteine (NAcTCVC) and be transported into the urine by multi drug resistance protein 2 (MRP2) and organic ion transporter (OAT) 1/3. NAcTCVC can also be activated by CYP3A to form the nephrotoxicant, NAcTCVC sulfoxide (Werner et al. 1996; Guyton et al. 2014). Alternatively, Aminoacylase III in renal proximal tubular cells can deacetylate NAcTCVC by converting it back to TCVC, which can be transformed to reactive species by β-lyase, FMO3 or CYP3A (Lash et al. 2014).

Once DCVC or TCVC have been activated by β-lyase to reactive species, they form mutagenic covalent DNA and protein adducts, generate acylated DNA bases, result in base substitution mutations, alter the ability of DNA to serve as a template for RNA and DNA polymerases, increase the expression of proto-oncogenes c-myc and c-fos, and increase single strand DNA breaks (Bhattacharya and Schultze 1972; Jaffe et al. 1985; Dekant et al. 1986; Vamvakas et al. 1993; Pahler et al. 1999; Dreessen et al. 2003; Lash et al. 2007; Irving and Elfarra 2013; Rusyn et al. 2014).

TCVG and TCVC can be measured in the blood and the nontoxic metabolite, NAcTCVC, can be measured in the urine. However, the production of reactive, toxic metabolites, including TCVT, DCTK, and TCVC sulfoxide cannot be readily measured by sampling blood and urine. While some of their protein adducts have been detected in blood or *in vitro* (Pahler et al. 1999; Barshteyn and Elfarra 2009), these reactive metabolites covalently bind to protein and /or DNA within tissues to produce toxic/mutagenic effects (Figure 2). For example, DNA adducts formed

by β-lyase activated ^{35}S-labled DCVC bind to and are retained on DNA bases (Bhattacharya and Schultze 1972; Dekant et al. 1986).

To predict PCE metabolism, PBPK modeling utilized the metabolite dichloroacetate (DCA) produced by both the oxidative and the GSH conjugation pathways and the non-toxic metabolite NAcTCVC produced by the GSH conjugation pathway (Chiu and Ginsberg 2011). This is problematic since it does not accurately estimate the flux through the GSH conjugation pathway and especially the production of toxicologically important reactive metabolites that covalently bind to DNA and protein within tissues. As discussed below, genetic polymorphisms at several loci in humans including *GSTT1*, *GSTM1*, and *CCBL1* influence the bioactivation of PCE metabolites to form the mutagenic and toxic reactive species, TCVT and DCTK and many of the enzymes discussed above show genetic polymorphisms that differ between ethnically divergent human populations.

Note that sensitive methods have also been developed for the simultaneous detection of TCE metabolites including trichloroacetic acid (TCA), dichloroacetic acid (DCA), dichlorovinyl glutathione (DCVG) and dichlorovinyl cysteine (DCVC) (Kim et al. 2009a). These methods have also been used to conduct a pharmacokinetic analysis of TCE metabolism in B6C3F1 mice (Kim et al. 2009b).

Variation in Xenobiotic Metabolism

There are many genetic, developmental, nutritional, tissue and sex-specific factors that determine the levels of enzymes involved in metabolizing xenobiotics. Figure 2 shows the enzymatic steps in the GSH conjugation pathway of PCE metabolism. Any variation or factors that increase GST

activity, and increase either β-lyase (CCBL) activity, AGAT/AGXT2 activity, FMO3 activity and/or CYP3A activity could increase production of highly toxic and mutagenic reactive metabolites (Lash et al. 2007). This could result in damage to several tissues with GGT activity, including kidney and pancreas (see pathways shaded in dark/red). In contrast, increasing NAT8 activity could increase the conversion of TCVC into NAcTCVC, which could reduce toxicity since this mercapturate is not toxic. Increasing the transporters, MRP2 and OAT 1/3 would tend to favor detoxicification by increasing NAcTCVC transport into the urine. However, increasing Aminoacylase III activity will convert NAcTCVC back to TCVC thereby increasing production of reactive and toxic compounds.

Cytochrome P450s (CYPs) oxidize and metabolically activate several xenobiotics, including polycyclic aromatic hydrocarbons (PAHs) and chlorinated compounds, such as TCE and PCE (Figure 1). CYP2B1/2 is induced by phenobarbital, glucocorticoids, ethanol, nicotine, and several xenobiotics, that interact with the constitutive androstane receptor (CAR) (Yamada et al. 2006). The genetic variant C1459T (R487C) has been associated with reduced hepatic enzyme levels, stability and activity (Miksys et al. 2003). Differences in CYP2B1/2 activity could potentially affect the rate of oxidative metabolism of PCE, subsequent production of toxic oxidative metabolites, as well as, the amount of PCE available for metabolism through the GSH conjugation pathway.

CYP3A enzymes are important in the detoxification of toxins, carcinogens, bile acids, steroid hormones and more than half of all prescribed medications (Keshava et al. 2004; van Herwaarden et al. 2007). CYP3A activity in human liver and intestine varies about 40-fold

among individuals (Lamba et al. 2002; Keshava et al. 2004). CYP3A activity in humans differs between genders and is influenced by genetic polymorphisms, compounds that induce its expression/activity, and compounds that inhibit its activity, such as grapefruit juice (Keshava et al. 2004; van Herwaarden et al. 2007; Li et al. 2015). Differences in CYP3A could potentially affect flux of PCE metabolites through the oxidative pathway (Figure 1) or the production of toxic sulfoxides in the GSH conjugation pathway (Figure 2).

Glutathione S-transferase (GSTs) form a class of enzymes with overlapping substrate specificities that play key roles in cellular detoxification. GST isozymes include α (alpha), μ (mu), π (pi), θ1 (theta 1) and θ2 (theta 2) and are coded by genes *GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*, and *GSTT2B*, respectively (Landi 2000; Polimanti et al. 2013).

GSTs function to detoxify many environmental chemicals. For example, *GSTM1* and *GSTP1* are thought to detoxify epoxides of PAHs (Bruning et al. 1997; He et al. 2014). Several GST loss of function variants, including deletion polymorphisms, alone and especially in combination, are associated with common disorders, including aplastic anemia, myelodysplastic syndrome (Lee et al. 2001), and cancers, including breast, bladder, acute myeloid leukemia (AML), and renal cell carcinoma (RCC) (Srivastava et al. 2005; Wu et al. 2013; Chirila et al. 2014; Huang et al. 2015). For halogenated ethenes such as TCE and PCE, GSTs, including GSTT1 and perhaps GSTM1 are the first step in the bioactivation of these compounds into reactive species (Bruning et al. 1997; Lash and Parker 2001; Lash et al. 2014). GSTT1 is primarily involved in the detoxification of aliphatic epoxides and activation of halogenated hydrocarbons including dichloromethylene, trichloroethylene and other halogenated methanes and ethanes (Thier et al. 1998; Landi 2000).

Low levels of TCE were conjugated by human GSTM1 and lower levels by human GSTP1, but not by human GSTA1 (Hissink et al. 2002). The liver is thought to be the main site for the GST-catalyzed conjugation of PCE to form TCVG, which is then released in to the blood, bile and hepatic-portal system where it can be picked up by many tissues, especially the kidney. GSTs in other organs, including kidney can also catalyze this conjugation reaction (Lash et al. 1998a; Lash et al. 2007). These studies suggest that GSTT1 and GSTM1 likely metabolize DCM, TCE and PCE.

Genes coding these enzymes show a number of polymorphisms with significant impacts on enzyme expression and catalytic activity. Deletion polymorphisms in human *GSTM1*, *GSTT1*, and *GSTT2* result in null alleles that lack GST activity when homozygous. Some single nucleotide/amino acid variants of GSTs, including *GSTT1* affect catalytic activity, while *GSTP1* affect catalytic activity and inhibition by heavy metals (Pal et al. 2000; Goodrich and Basu 2012).

Glutathione conjugation of halomethanes including dichloromethane in humans is controlled by *GSTT1* genotype, with *GSTT1* null (-/-) homozygotes showing no conjugation, *GSTT1* +/+ homozygotes showing high conjugation and *GSTT1* +/- heterozygotes showing intermediate conjugation (Hallier et al. 1994; Thier et al. 1998; Wiebel et al. 1999). Although the GST isozyme(s) responsible for the conjugation of PCE has not been determined, similar chlorinated substrates are metabolized by GSTT1. The genetic evidence described above for conjugating dichloromethane in humans, suggests that GSTT1 and perhaps GSTM1 may metabolize TCE and PCE.

The presence of one or more functional/wild type alleles at *GSTT1* (Bruning et al. 1997; Moore et al. 2010) and *GSTM1* (Bruning et al. 1997) is associated with increased RCC risk in chlorinated solvent/TCE exposed workers, as well as in pesticide exposed workers (Buzio et al. 2003; Karami et al. 2008). An evaluation of RCC risk in workers with long-term occupational exposure to chlorinated solvents, including TCE, showed that individuals with functional alleles present at *GSTT1* (Odds Ratio (OR) 4.16, $p<.05$) and at *GSTM1* (OR 2.74 $p<0.02$) had significantly higher risk of RCC, over those with homozygous null (-) alleles at these loci (Bruning et al. 1997). TCE exposed individuals with functional alleles at both *GSTM1* and *GSTT1* showed the greatest RCC risk (Bruning et al. 1997).

Moore et al. 2010 also examined RCC risk in a central/eastern European population with occupational exposure to chlorinated solvents, including TCE. Hours of chlorinated solvent exposure and average intensity of chlorinated solvent exposure were associated with increased RCC risk ($p <0.04$). Among TCE-exposed workers, RCC risk is increased for individuals with one or more *GSTT1* + alleles ($p< 0.02$), and for individuals with certain polymorphisms in *CCBL1*, the gene coding for β-lyase ($p <0.01$) (Moore et al. 2010). This study shows that genotype at both *GSTT1* and at *CCBL1* influenced RCC risk in TCE-exposed workers.

The TCE exposed workers characterized by Moore et al. (2010) are from central and eastern Europe while those characterized by Brüning et al. (1997) are from Germany south of Ruhr and likely had little ethnic diversity. The frequency of gene polymorphisms likely associated with RCC risk is also examined in more diverse ethnic populations, including those genotyped by the International Haplotype Mapping Project (Hapmap 2016).

Comparisons of polymorphisms at several GST loci show a high genetic diversity in GST isozymes among human ethnic populations (Polimanti et al. 2013). Differences in gene frequency at GST loci are important since they code for functional inter-ethnic differences in detoxification systems (Polimanti et al. 2013). The frequency of individuals that are homozygous null genotype *at GSTM1* differs between ethnic populations (Iorio et al. 2014; Karaca et al. 2015). The frequency of individuals with at least one copy of an active (+) *GSTM1* ranges from about 41% in individuals with central/western European ancestry, up to 77 to 80% in individuals with African ancestry (Karaca et al. 2015).

The frequency of individuals with the *GSTT1* homozygous null genotype differs between ethnic populations, with a frequency of 9 to 22% for Mexican-Americans and 40 to 65% for Asian populations (Josephy 2010; Iorio et al. 2014; Karaca et al. 2015; Polimanti et al. 2015). See Table 1. The frequency of individuals with at least one *GSTT1* + allele ranges from about 35 to 60% in Chinese, 40 to 60% in Koreans and Japanese, 65 to 70% in Africans, 76 to 89% in European Caucasians, 77% in Brazilians, 78% in African-Americans, 78 to 91% in Mexican-Americans and up to 93% in some Turkish populations (Nelson et al. 1995; Josephy 2010; Iorio et al. 2014; Karaca et al. 2015; Polimanti et al. 2015). For eleven ethnic populations genotyped in the International HapMap project shown in Table 1 the frequency of individuals with at least one *GSTT1* + allele ranged from 55% in Han Chinese up to 89% in Caucasians of northern/western European ancestry (Karaca et al. 2015; Hapmap 2016).

Table 1 also shows the estimated frequency of individuals that are wild--type at both *GSTM1* and *GSTT1*, assuming these loci are in Hardy-Weinberg equilibrium. Individuals with active alleles

at both *GSTM1* and *GSTT1* ranged from 26 to 30% in Asians, 36 to 37% in European Caucasians, 42% in Mexican-Americans, 55% in Gujarati Indians, and up to 53 to 62% in Africans and African-Americans.

GSTM1 + may also increase risk of RCC by inhibiting the apoptosis signal-regulating kinase 1 (ASK1) signal transduction pathway. ASK1 is a mitogen activated protein kinase (*MAP3K5*) that activates downstream kinases such as P38 (Cho et al. 2001; Dorion et al. 2002; Huang et al. 2015). When cells are exposed to stress or reactive oxidants, ASK1 activates P38, which induces apoptosis. However, the inhibition of ASK1 by *GSTM1* + could inhibit apoptosis and lead to an increased risk of RCC (Cho et al. 2001; Dorion et al. 2002; McIlwain et al. 2006; Huang et al. 2015). *GSTM1* + alleles may also increase the risk of cancer caused by the reactive and mutagenic oxidants, TCVT, DCTK, TCVC sulfoxide and NAcTCVC sulfoxide generated through the GST/β-lyase PCE metabolism pathway (Huang et al. 2015). *GST* mutations also affect the rate of progression of cancers and responses to chemotherapy (McIlwain et al. 2006; Huang et al. 2015).

These and other studies on diverse human populations show that a large amount of inter-ethnic diversity is present in the allelic frequencies of several GST loss of function variants, and that ethnicity is likely to influence GST-disease associations (Polimanti et al. 2013).

γ-Glutamyl transpeptidase or gamma glutamyl transferase (GGT) is a cell-surface enzyme that cleaves γ-glutamyl bonds of GSH and GSH S-conjugates and is induced by oxidative stress. GGT activity is higher in PCE-exposed dry cleaning workers (Lash and Parker 2001; Zhang and

Forman 2009; Wickham et al. 2013) and several-times higher in fetuses and infants than in adults (Moniz et al. 1984; Cabrera-Abreu and Green 2002).

Beta lyase (β-lyase)**,** also known as cysteine conjugate-beta lyase 1 or kynurenine---oxoglutarate transaminase 1 (*KAT1*) is an enzyme that is coded by the *CCBL1* gene in humans. This enzyme is responsible for the metabolism of cysteine conjugates of halogenated alkenes and alkanes, including TCE and PCE to form toxic reactive metabolites (Figure 2) (Lash and Parker 2001; Lash et al. 2014). Multiple transcripts (splice variants) that encode different isoforms have been identified for this gene (NCBI Genome Viewer 2016). Gliomas in rat brain are significantly elevated in the carcinogenicity bioassay of PCE (NTP 1986), and there is potential for formation of reactive metabolites in sites other than kidney, e.g., the brain (Lash and Parker 2001; USEPA 2012). A sample of 20 US kidney donors differed 4-fold in β-lyase activity using the similar cysteine-S-halogen alkene substrate, fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl ether (FDVE) (Gul Altuntas and Kharasch 2002). However, the ethnic background of these donors and the β-lyase activity of diverse ethnic populations is unknown. Almost 5-fold variation in β-lyase activity is observed among a limited number of human kidneys (McCarthy et al. 1994). Both of these studies support substantial variation in human β-lyase activity. The presence of moderate GGT activity and relatively high β-lyase activity in the pancreas is also significant since workers involved in metal degreasing or dry cleaning had significantly higher pancreatic cancer risk, and worker exposure to TCE or PCE is suggestively associated with pancreatic cancer risk (Ojajarvi et al. 2001).

Genetic polymorphisms in *CCBL1* are significantly associated with RCC risk in chlorinated solvent/TCE exposed workers in central Europe (Moore et al. 2010). Approximately 14 to 30% of the central European population sampled by Moore et al. (2010) possesses at least one *CCBL1* allele associated with increased β-lyase activity. Table 1 shows the genotype frequencies of one of the *CCBL1* (β-lyase) single nucleotide polymorphisms (SNPs) associated with RCC risk by Moore et al. (2010) in 11 different human ethnic populations characterized by the International Haplotype Mapping Project (Hapmap 2016).

The HapMap data set shows that the percentage of individuals with least one high risk *CCBL1* allele identified by Moore et al. (2010) differs between ethnic populations, ranging from about 38% in Caucasians (Utah residents with Northern/Western European Ancestry, i.e., CEU), 40 to 52% in Asians, 51% in Tuscan Italians, 60% in Gujarati Indians, and up to 79 to 96% in African-Americans and Africans (Hapmap 2016). The right-most column in Table 1 shows the estimated percentage of individuals with high risk TCE-induced RCC alleles at both *GSTT1* and *CCBL1*. It ranges from 23-31% in Asians, 34% in CEU Caucasians, 42% in Tuscan Italians, 48% in Gujarati Indians, and up to 55 to 64% in Africans and African-Americans (Hapmap 2016). Note that the *GSTT1* and *CCBL1* alleles associated with susceptibility to TCE-induced RCC are common in several ethnic populations and are not rare alleles.

In addition to CCBL1/β-lyase, six other mammalian aminotransferases, including L-alanine-glyoxylate aminotransferase (AGAT) I and II, are known to catalyze β-lyase reactions with toxic halogenated cysteine S-conjugates (Cooper et al. 2003; Human Protein Atlas 2016). AGAT II (also known as AGXT2) has β-lyase activity on several halogenated cysteine S-conjugates,

including DCVC and is thought to also contribute to the activation and toxicity of DCVC and TCVC (Cooper et al. 2003).

AGAT II is coded by the *AGXT2* gene on human Chromosome 5. Some humans express this enzyme, while others do not (Cooper et al. 2003). *AGXT2* shows over 40 SNPs, one of which, rs37369 was identified as associated with hyper-β-aminoisobutyric aciduria in a genome-wide association study (GWAS) of metabolic traits in humans (Suhre et al. 2011; Hapmap 2016). Hyper-β-aminoisobutyric aciduria is one of the most common Mendelian metabolic variants in man (Suhre et al. 2011). Ethnic populations differ markedly in gene frequency at rs37369. The frequency of individuals with at least one high *AGXT2* activity C allele ranges from approximately 55% in Japanese and Yoruban Nigerians up to 99 to 100% in Caucasians and Tuscan Italians (Hapmap 2016) (Table 2). Additional studies are needed to determine the effect of *AGXT2* on β-lyase activity and susceptibility to TCE and PCE-induced toxicity. However polymorphisms in *AGXT2* may also have the potential to contribute to ethnic variability in β-lyase activation of PCE and TCE to reactive metabolites.

Genetic variation in N-acetyltransferases, including Cysteinyl-S-conjugate N-acetyltransferase (NAT8) could potentially influence the production of reactive species in the PCE GSH conjugation pathway (Pinto and Dolan 2011) (Figure 2). *NAT8* codes for cysteinyl-S-conjugate N-acetyltransferase, the microsomal enzyme that catalyzes mercapturate formation, including the conversion of TCVC to NAcTCVC (Veiga-da-Cunha et al. 2010). Since NAcTCVC is excreted in the urine and cannot be used as a substrate for β-lyase, this enzyme is an important detoxification step in this pathway.

A study of donated human kidneys showed that rates of N-acetylation of a similar cysteine-S-halogen alkene substrate differed 70-fold among kidneys from 20 humans (Gul Altuntas and Kharasch 2002). While the genotype and ethnic background of these kidney samples is unknown, these data show a relatively large amount of variation in cysteinyl-S-conjugate N-acetyltransferase activity between humans.

Genetic polymorphisms in the 5' upstream promoter of *NAT8* are common and have been associated with effects on blood pressure, estimated glomerular filtration rate (eGFR) and nephrotoxicity response (Juhanson et al. 2008). A missense mutation in *NAT8*, R149K, suppresses activity of this enzyme (Veiga-da-Cunha et al. 2010). *NAT8* is expressed almost exclusively in the kidney with much lower levels in the liver (Human Protein Atlas 2016). A genome-wide association study in African-Americans shows that a missense genetic polymorphism in *NAT8* (rs13538) with a gene frequency of 0.48 is related to N-acetylornithine, N-acetyl-1-methylhistidine and creatine metabolite levels, estimated glomerular filtration rate (eGFR) and increased chronic kidney disease (Suhre et al. 2011; Yu et al. 2014). GWAS in a large Caucasian population also shows that NAT8 rs13538 with a minor allele frequency of 0.23 is associated with eGFR (Kottgen et al. 2010). The frequency of the minor (T) allele at this locus differs between HapMap ethnic populations ranging from 0.432 in Yoruban Nigerians, 0.792 in Caucasians and up to 1.0 in Japanese.

Exposure of human renal proximal tubule cells to 1 micro molar DCVC *in vitro* resulted in several changes in gene expression, including a greater than 2-fold reduction in *NAT8* expression (Lock et al. 2006), which might reduce detoxification. Given these genetic polymorphisms and

observations, additional studies are needed to determine the effect of *NAT8* SNPs on susceptibility to PCE and TCE-induced toxicity and cancer.

Aminoacylase III is coded by *ACY3*. In the renal proximal tubule cell this enzyme deacetylates cysteine S-conjugate mercapturates produced by the GSH conjugation pathway of TCE and PCE metabolism, reactivating it (Lash et al. 2014). Mouse aminoacylase III deacetylates NAcDCVC and NAcTCVC (Newman et al. 2007). In the case of PCE metabolism, Aminoacylase III converts NAcTCVC back into TCVC which can be converted into toxic reactive species by β-lyase (Figure 2).

The rates of deacetylation of a similar cysteine-S-halogen alkene substrate differed 7.4-fold amongst kidneys from 20 humans (Gul Altuntas and Kharasch 2002). In these samples, deacylation (i.e. reactivation) of the cysteine S-conjugate haloalkenes, predominated over N-acetylation (i.e. detoxification). The ratio of deacylation to N-acetylation ranged from a low of 2-fold to a maximum of 54-fold, depending on the individual. Although the ethnic diversity and genotype of these individuals is unknown, this study shows considerable variation in the deacylation reaction, the N-aceylation reaction and the ratio of these two reactions between individuals (Gul Altuntas and Kharasch 2002; National Academies Institute of Medicine 2015). Several genetic polymorphisms in ACY3 differ between ethnic populations and have been associated with pharmacological response phenotypes (Hiltunen et al. 2015; Hapmap 2016).

Metabolites of TCE/PCE are transported from blood to urine by multi drug resistance associated protein 2 (MRP2), as well as organic anion transporter (OAT)1 and OAT3

NAcDCVC, (and NAcTCVC), are transported in mouse renal proximal tubule cells into the urine by MRP2 (Tsirulnikov et al. 2010). OAT1 and perhaps OAT3 are also involved in transport of DCVC and TCVC and their mercapturates (Groves et al. 2003; Lash et al. 2014). These transporters influence toxicity of TCE and PCE cysteine S-conjugate metabolites by determining whether they are excreted into the urine, or remain in the renal proximal tubule cell where they may be activated by FMO3 or CYP3A to toxic sulfoxides (Tsirulnikov et al. 2010; Lash et al. 2014). If not transported into the urine, NAcDCVC and NAcTCVC can also be reactivated by Aminoacylase 3 to reform DCVC or TCVC, respectively. Genetic polymorphisms in *MRP2*, *OAT1 (SLC22A6*) and *OAT3* (*SLC22A8*) affect levels of these transporters and the transport of many drugs and xenobiotics (Riedmaier et al. 2012; Lash et al. 2014). The 5' regulatory region of human *SLC22A8* shows several polymorphisms in the proximity of steroid and other regulatory elements, which differ between ethnic populations (Bhatnagar et al. 2006). The frequency of the G allele at *SLC22A8/OAT3* 1182G>C ranges from a low of 0.357 in Northern Saharan Africans, 0.555 in Northern Europeans, and up to 0.90 in Ashkenazi Jews (Bhatnagar et al. 2006). Given these observations, additional studies are needed to determine which polymorphisms influence the retention versus excretion of DCVC, TCVC and their N-acetylated metabolites and kidney toxicity/mutagenesis.

Activation of TCVC

In addition to activation by β-lyase, TCVC can also be activated by FMO3 or by CYP3A to form the potent nephrotoxicant, TCVC sulfoxide (Elfarra and Krause 2007; IARC 2014). After the non-toxic mercapturate, *N*-acetyl trichlorovinyl cysteine (NAcTCVC) is formed, it can be

excreted in urine, or converted by CYP3A to form the highly toxic and reactive sulfoxide NAcTCVCS (Lash and Parker 2001; Lash et al. 2014). Genetic variants or conditions that increase FMO3 or CYP3A activity could potentially increase the production of toxic reactive sulfoxides, including in tissues lacking β-lyase activity.

The activity of CYP3A differs up to about 40-fold between individuals (Lamba et al. 2002; Keshava et al. 2004; van Herwaarden et al. 2007). Genetic polymorphisms in *CYP3A* and factors that induce this enzyme are discussed above and below.

FMO3 activity and liver FMO3 protein levels differ 20 to 40-fold between individual humans (Cashman and Zhang 2002; Nagashima et al. 2009; NCBI Gene ID 2016). The expression of this enzyme is tissue specific, and inter-individual differences in FMO3 activity appear to be genetic rather than inducible (Cashman and Zhang 2002). *FMO3* shows common genetic polymorphisms between ethnically divergent human populations, including Caucasians, Africans, Asians and Hispanics. Estrogen also inhibits FMO3 expression in mice and humans (Esposito et al. 2014).

In addition, according to the NCBI Variation Viewer/1000 Genomes Project Database, there are many more variants at *CCBL1, AGXT2; NAT8; ACY3; ABCC2* (MRP2); *SLC22A6* (OAT1); *SLC22A8* (OAT3); *FMO3; HNF-4*, and *CYP3A4* with minor allele frequencies of greater than 0.05 in ethnically diverse populations. Such genetic polymorphisms are also significant given that of all human polymorphisms, roughly a third are shared across all continents, roughly a third are shared within a continental origin area, and the remaining portion are private to a continental area or unique to a specific ethnic population (The 1000 Genomes Project Consortium 2015). Thus, studies such as Brŏning et al. (2007) and Moore et al. (2010) that examined the association

of TCE-induced cancer risk with genotype within a mainly Caucasian population may have missed genetic polymorphisms that are only found in other ethnic populations.

Flux Through GSH Conjugation

Most estimates of the overall flux of PCE metabolism through GSH conjugation pathway have been based on very limited TCE or PCE data from a very small number of rodent strains and individual humans (Lash and Parker 2001; Lash et al. 2007; Chiu and Ginsberg 2011). Some studies have considered the initial GST-catalyzed GSH conjugation step, others measured activity of β-lyase or NAT, and others measured TCA and detoxified urinary mercapturate. However, the reactive metabolites of the GSH conjugation/β-lyase pathway bind to DNA and protein and cannot be readily quantitated.

There has been debate in the literature regarding the rates of GSH conjugation of TCE and PCE by rodents and humans (Lash et al. 2014). The Lash laboratory determined rates of glutathione conjugation by exposing animals, humans, cells, cytosols or microsomes to TCE or PCE. They then determined DCVG or TCVG produced by derivatizing these metabolites followed by HPLC separation and quantitation (Lash et al. 1998a; Lash et al. 1998b; Lash et al. 1999a; Lash et al. 2007). In contrast, the Green laboratory incubated cytosol or microsomal fractions with ^{14}C-TCE or ^{14}C-PCE and then quantitated products by HPLC separation followed by scintillation counting (Green et al. 1990; Green et al. 1997).

Rates of TCE conjugation by F344 rat and B6C3F1 mouse liver cytosols measured by Lash et al. (1998b) are about 2 orders of magnitude greater than those estimated by Green et al. (1997) in the same rat and mouse strains. Rates of PCE conjugation by rat and mouse liver cytosols and

microsomes measured by Lash et al. (1998a) are also 1 to 2 orders of magnitude greater than those estimated by Green et al. (1990) in the same strains. Lash et al. (1998b) validated their methods and showed that the rates of DCVG formation in rat liver and kidney cells are in good agreement with those of cytosolic and microsomal fractions of the corresponding tissues. Lash et al. (1999a) validated their methods and measured rates of DCVG formation *in vitro* by 20 human liver cytosols that are about 9,000 to 22,000-times greater per mg protein per minute than the human liver cytosol reported by Green et al. (1997). Rates of TCVG formation by human liver cytosol and microsomes reported by Green et al. (1990) are below detection limits and were at least an order of magnitude lower than that of rats in this study.

TCE conjugation rates in subcellular fractions from 20 human livers measured by Lash et al. (1999a) are about 950 to 2300-times greater per mg protein than the rates of PCE conjugation in human liver cytosol estimated by Green et al. (1990) and are about 800 to 5000-times greater than the rates of PCE conjugation in liver microsomes proposed by Green et al. (1990). This comparison of liver GSH conjugation rates of PCE and TCE is relevant since they involve the same GST enzymes, and since rates of PCE conjugation have been shown to be similar or slightly higher than rates of TCE conjugation in rat hepatocytes (Lash et al. 2007).

The methods for measuring GSH conjugation and very low estimates of GSH conjugation of Green et al. (1990) are questionable for several reasons (Lash et al. 2014). Measuring TCVG is challenging since TCVG is unstable and can also be converted to subsequent metabolites in the pathway (Lash and Parker 2001; Lash et al. 2007). The method used by Green et al. (1997) to estimate DCVG formation from ^{14}C-TCE shows an artifact due to a ^{14}C-TCE contaminant that

spontaneously reacts with GSH to form DCVG in the absence of cytosol or microsomes (See Green et al. 1997; Table 3 and Discussion). The data show that even after ^{14}C-TCE purification, addition of GSH to buffer control incubations resulted in rates of DCVG formation similar to incubations with liver microsomes or kidney cytosols, and greater than incubations with kidney microsomes (Green et al. 1997). Due to this artifact, the method of Green et al. (1997) for estimating GSH conjugation of TCE is questionable. Similarly, since validation data is not provided, the ^{14}C tracer method of Green et al. (1990) for estimating GSH conjugation of PCE is also questionable.

The GSH conjugation data of Lash et al. (2007) in rat hepatocytes and kidney cells provides further support for the higher estimate of GSH conjugation. In the presence of 2 mM PCE, 5 mM GSH, and 0.25 mM acivicin to inhibit further metabolism of GSH S-conjugates, rat hepatocytes produced about 10 nmol TCVG per 30 minutes per 10^6 cells while rat kidney cells produced about 1.2 nmol TCVG per 30 minutes per 10^6 cells. Parallel incubations with TCE rather than PCE, show that the rates of PCE conjugation slightly exceeded the rates of TCE conjugation (Lash et al. 2007).

Levels of DCVG in blood following a 4-hour inhalation exposure to TCE, provides more evidence for GSH conjugation in humans (Lash et al. 1999b). Following inhalation exposure to 100 ppm TCE, blood levels of DCVG peaked at 46.1 ± 14.2 nmol/ml in males, with about half of the males showing relatively low levels of DCVG similar to that found in females. The remainder of the males showed higher levels of DCVG in blood up to 94 nmol/ml, suggesting that this trait may be influenced by a genetic polymorphism (Lash et al. 1999b). Despite

micromolar concentrations of DCVG in blood of several human subjects, NAcDCVC is only routinely detectable in the urine of the male with the highest levels of blood DCVG (Lash et al. 1999b).

Overall Flux Through β-lyase Versus Acetylation

Green et al. (1997) also determined the Vmax and Km of renal β-lyase (CCBL) and N-acetyl transferase (NAT) for DCVC. Although the number of human kidneys sampled is unclear, metabolic clearance (V_{max}/K_m) of DCVC through β-lyase were similar in B6C3F1 mice and human(s). These clearance rates are about 10-fold lower than in F344 rats (Green et al. 1997, Table 4). The metabolic clearance of DCVC through NAT is about 59 and 43-fold higher in rats and mice, respectively, than in humans (Green et al. 1997, Table 4). These data also suggest that the fraction of the flux from DCVC (and regenerated DCVC) going through β-lyase to produce toxic, mutagenic, reactive metabolites is greater in humans than in mice.

Using TCVC as substrate, Green et al. (1990) examined β-lyase activity in kidney cytosol of male and female F344 rats, B6C3F1 mice, as well as of 3 human males and 4 human females. In mice and rats, the Km of β-lyase for TCVC is similar to that reported for DCVC by Green et al. (1997). However, in humans the β-lyase Km (2.53 mM in males and 2.67 mM in females) for TCVC is about 4-fold lower than for DCVC. The estimated average metabolic clearance (Vmax/Km) of TCVC by β-lyase in humans is similar to that of B6C3F1 mice but about 10-fold lower than that of F344 rats. However, the β-lyase Vmax of 0.64 +/- 0.54 nmol/min/mg protein in 4 women shows a greater coefficient of variation than other groups. These data suggest that β-

lyase V_{max} is near zero for two women and near the mean of B6C3F1 mice for the other two women.

Using the cystene-S-halogen alkene, FDVE, as substrate, the activity of β-lyase, CCNAT (the acelyation reaction) and the deaceylation reaction varied 4-fold, 70-fold, and 7-fold, respectively, among 20 human kidney samples (Gul Altuntas and Kharasch 2002). Analysis of enzyme activities show that β-lyase catalyzed metabolism to toxic reactive products is heavily favored over N-acetylation to the non-toxic mercapturate. The ratio of β-lyase catabolized metabolism to N-acetylation metabolism of cysteine S-conjugates, ranged from 3-fold to 146-fold between individuals and averaged 32-fold (Gul Altuntas and Kharasch 2002). Since no information was reported on the ethnic background or genotypes of these 20 human kidney samples, the magnitude of differences between ethnically diverse populations is unknown.

The study of Altuntas and Kharasch (2002) indicates that estimating the production of toxic reactive products of the GST conjugation/β-lyase pathway by measuring the mercapturate in urine is likely to underestimate production of toxic reactive species by β-lyase. Since the DCA formed also does not account for reactive species bound to DNA or protein, measuring NAc-TCVC and DCA in blood or urine is also not sufficient to estimate toxic reactive species produced by the GSH conjugation/β-lyase pathway.

Volkel et al. (1998) exposed 6 humans and Wistar rats for 6 hours in inhalation air chambers to 10, 20 or 40 ppm PCE. Then they examined the cumulative urinary excretion of NAcTCVC, and TCA over 35 hours. For a given inhalation exposure, rats converted considerably more PCE to NAcTCVC and TCA than humans (Volkel et al. 1998). Nevertheless, of the PCE that is

metabolized, humans converted a higher portion to NAcTCVC than did rats. These data show that cumulative urinary NAcTCVC averaged about 1 percent of cumulative urinary TCA in 6 humans (range of 0.93 to 1.12%) and about 0.25% of cumulative TCA in rats (range of 0.18 to 0.35%). Although this study did not examine the toxic and mutagenic reactive metabolites of the PCE GSH conjugation pathway, it does show that of the PCE metabolized, humans metabolized a greater percent through the GSH conjugation pathway to NAcTCVC than rats.

Urinary metabolites, including NAcDCVC and TCA are also measured in 4 workers following occupational exposure to TCE and in rats and mice following oral gavage with 50 mg/kg TCE (Birner et al. 1993). Due to varying exposures, the ratio of urinary NAcDCVC/TCA is most informative, i.e., the ratio of TCE metabolized and detoxified through N-acetylation in the GSH conjugation pathway versus metabolized to TCA in the oxidative pathway. Urinary NAcDCVC is 1 to 7% of urinary TCA in mice and rats, 3 to 6% of urinary TCA in three workers and 33% of urinary TCA in another worker.

Toxicodynamic Responses in Genes Controlling DNA Repair

Polymorphisms in several DNA repair genes, including oxyguanine glycosylase 1 (*OGG1*), X-ray repair cross-complementing protein 1 (*XRCC1),* and *BRCA2* have been associated with cancer risk (Goode et al. 2002; Zhi et al. 2012) and differ between individuals and between diverse ethnic populations (Pramanik et al. 2011).

Polymorphisms in *XRCC1*, including an Arg399Gln variant and a -77T>C SNP affect DNA repair, and have been considered for potential use in risk assessment (Ginsberg et al. 2011). Individuals that are homozygous (Gln/Gln) at *XRCC1* codon 399 can have 3--4-fold lower

capacity to remove DNA adducts and oxidized DNA damage. About 10% of Caucasians and Asians are homozygous for this variant (Ginsberg et al. 2011). Another variant, Arg194Trp, protects against genotoxic effects, is rare in Caucasians and African-Americans but about 7% of Asians are homozygotes.

As discussed above, reactive species produced by the PCE GSH conjugation pathway have been shown to produce DNA adducts and mutations (Bhattacharya and Schultze 1972; Jaffe et al. 1985; Dekant et al. 1986; Vamvakas et al. 1993; Dreessen et al. 2003; Lash et al. 2007; Irving and Elfarra 2013). Thus, genetic polymorphisms controlling repair of PCE-induced adducts/mutations differ between ethnic populations and are likely associated with PCE-induced toxicity and cancer risk. Genetic polymorphisms controlling apoptosis are also likely to affect toxicodynamic responses, as discussed above.

Studies on Toxicokinetic and Toxicodynamic Responses to TCE and PCE

The variability of TCE metabolism is examined in 17 mouse strains (Chiu et al. 2014). Since TCE and PCE appear to be metabolized by the same pathways and many of the same enzymes, including *CYP, GSTT1, GGT, CCBL1/β-lyase, NAT8, ACY3*/Aminoacylase and *FMO3*, and transporters including *MRP2* and *OAT1/3*, the study on TCE metabolism and mouse genetic variation provides an initial model that can be evaluated for genetic variation in PCE metabolism (Chiu et al. 2014). PBPK modeling of inter-strain variability in TCE metabolism in diverse mouse strains shows considerably more variability between strains in the GSH conjugation pathway than in the oxidative metabolism pathway (Bradford et al. 2011; Chiu et al. 2014). TCE metabolized through the GSH conjugation pathway showed a high degree of variability both

between genetically divergent mouse strains and between individual humans (Lash et al. 1999a; Lash et al. 1999b; Bradford et al. 2011; Chiu et al. 2014). Diverse mouse strains also differed greater than 10-fold in the ratio of metabolites through the early steps in the GSH conjugation pathway versus through the CYP pathway (Chiu et al. 2014). Strain differences in the latter steps of the GSH conjugation pathway including production of reactive species through β-lyase, FMO3 and CYP3A or detoxification by N-acylation by NAT8 have not been examined to date, and needs to be determined.

Studies in humans have shown that polymorphisms in *CCLB1*/β-lyase are associated with RCC risk in TCE exposed workers (Moore et al. 2010). Since TCE and PCE are metabolized by the same enzymes in the GSH conjugation/β-lyase pathway, these and related studies provide a potential model for the genetic variation in PCE metabolism.

Toxicokinetic and toxicodynamic responses to oral PCE are also examined in a more diverse set of 45 Collaborative Cross mouse strains (Churchill et al. 2004; Cichocki,Furuya, et al. 2016; Cichocki et al. In Press). Strains show about 8-fold differences in PCE and TCA levels in the liver and about 3.5-fold differences in TCA levels in kidney and serum. Strains also differed in PCE-induced toxicity responses. This study shows large differences in PCE-induced TCA levels across genetically diverse mouse populations, but indicates that TCA levels do not account for hepatic PPARα, triglyceride and steatosis responses and toxicity across genetically diverse mouse strains. This study shows that strains differ both in the levels of toxic metabolites produced from PCE, and the toxicodynamic responses to these metabolites. Additionally, this

study demonstrates the importance of considering both the toxicokinetic responses and the toxicodynamic responses of genetically diverse populations.

An RNA-sequencing approach was also used to evaluate the genetic variation in the adult male hepatic expression of Phase I and II metabolism genes across 29 diverse Collaborative Cross (CC) mouse strains (Churchill et al. 2004; Nachshon et al. 2016). Comparing gene expression data for genes involved in Phase I and II metabolism across CC strains with known pathways of xenobiotic metabolism allowed mapping expression quantitative trait loci (eQTL) and identifying potential underlying genetic differences in metabolism of several xenobiotics. This study identified cis-acting hepatic eQTL as being possibly involved in genetic differences in the oxidative metabolism of TCE at *Cyp2c40* and *Cyp2c44*, and in the GST conjugation pathway at *Gsta2*, *Gstm6*, *Mgst3*, *Ccbl1*, and *Ccbl2* (Nachshon et al. 2016). The eQTL observed at *Fmo1* and *Nat8* may also be involved in genetic differences in TCE/PCE metabolism. This study documents genetic variation between strains of mice in the expression of several Phase I and II metabolism genes involved in TCE/PCE metabolism. This study also provides the foundation for future studies to determine if these eQTL cause differences in metabolism of xenobiotics, and effect susceptibility to xenobiotics including TCE and PCE.

Sensitivity of Fetuses, Infants and Children

Children and especially fetuses and infants, can be much more susceptible to bioactivated environmental toxicants than adults due to higher exposures per unit body weight and particularly due to differences in the developmental timing of the acquisition of specific Phase I and Phase II enzymes (Fanucchi et al. 2000). The expression of many different CYP RNAs is

low in fetuses but increases markedly during postnatal and juvenile development in C57Bl/6J (B6) strain mice (Peng et al. 2013). Several CYP enzymes involved in the oxidative metabolism of TCE and PCE including CYP2E1 and CYP2B1/2 and CYP3A1 are very low in human and rodent fetal tissues and increase markedly postnatally to the higher levels found in adults (Johnsrud et al. 2003; Day et al. 2006). While CYP2E1 in human liver increases markedly in neonates and infants, the increases are highly variable, with about a 80-fold range among neonates (Johnsrud et al. 2003). CYP2E1 levels during this period are also influenced by genotype and ethnicity (Johnsrud et al. 2003). Of human liver CYP3A isozymes, CYP3A7 is expressed prenatally and declines soon after birth, while CYP3A4 increases from infancy to adulthood (de Wildt et al. 1999). Nevertheless, fetal and infantile CYP3A activity differs between individuals, with CYP3A7/CYP3A5 genetic variants differing in frequency between ethnic groups and in catalytic activity toward certain xenobiotics (Rodriguez-Antona et al. 2005; Stevens 2006).

In contrast, several Phase II enzymes involved in the bioactivation of TCE/PCEs through the GSH conjugation pathway show a somewhat different pattern of expression during perinatal and postnatal development that is not coordinated with the expression of Phase I enzymes. Expression profiling of hepatic RNAs in male B6 mice from -2 to 60 days of age shows greatest expression of *Gstm5, Gstt2* and *Ggt1*in the perinatal period, greatest expression of *Ccbl2* and *Fmo3* in adolescents, and greatest expression of many other genes including *Ccbl1, Nat 1, Nat 8* and several *Gstt* and *Gstm* isozymes in the juvenile or adult period (Lu et al. 2013). Although *Nat8* plays a critical role in detoxification in the GSH conjugation pathway (See Figure 2), the data of Lu et al., 2013 show that this gene is one of the last in this pathway to be expressed

during development. Human fetal liver contains levels of GSTs that approach or exceed that of adults. These enzymes metabolize several halogenated compounds, epoxides and α, β-unsaturated compounds (Scheuplein et al. 2002). Levels of GGT are also higher in fetuses and infants than in adults (Moniz et al. 1984; Cabrera-Abreu and Green 2002). Thus, the patterns of gene expression during development raise concern that fetuses, neonates and infants may be more susceptible than adults to xenobiotics including TCE/PCE that undergo bioactivation due to potentially limited detoxification through the oxidative pathway and potentially elevated bioactivation through the GSH conjugation pathway.

The many genetic and developmental factors controlling the GSH conjugation pathway of TCE/PCE metabolism may also influence susceptibility to TCE/PCE induced suppression of host defenses, compromised pregnancies, and infant morbidity and mortality. Human exposure to TCE has also been associated with low birth weights and with cardiac/conotruncal defects (Forand et al. 2012). Interestingly, exposure to DCVC but not TCA, inhibited the release of several cytokines from human placental membranes in response to Group B streptococcus (GBS) infection (Boldenow et al. 2015). The inhibition of cytokine release by DCVC is important, since cytokine release plays a critical role in recruiting an immune response to bacterial infection (Boldenow et al. 2015). GBS and other genitourinary tract infections are a major cause of premature delivery, prenatal and neonatal infections, and infant morbidity and mortality (Cram et al. 2002; Chan et al. 2013; Boldenow et al. 2015).

Differences between Genders in Xenobiotic Metabolism and Susceptibility

Several studies have reported differences between male and female rodents in the rates of metabolism and susceptibility to PCE, with males showing higher cancer incidence than females (NTP 1986; Lash et al. 2007; USEPA 2012; OEHHA 2016a). Expression of several enzymes involved in TCE and PCE metabolism, including CYP2E1 and CYP3A are regulated in a sex-dependent manner (Mugford and Kedderis 1998; Penaloza et al. 2014; Li et al. 2015). Liver oxidative metabolism of TCE is higher in male than in female rats (Lash et al. 2007; Lash et al. 2014). Human males show 3.4-fold higher levels of DCVG in blood than females following TCE inhalation exposure (Lash et al. 1999b). DCVC metabolism appears to be more rapid in male than female rats (Lash et al. 2014). PCE-exposed male rats show higher cumulative urinary excretion of TCA and NAcTCVC than female (Volkel et al. 1998). The cytotoxicity of PCE and TCVG is greater in rat kidney cells isolated from males than from females (Lash et al. 1998a; USEPA 2012). Estrogen also inhibits FMO3 expression in mice and humans (Esposito et al. 2014). Thus, the literature shows evidence for differences between sexes in the toxicokinetics of PCE metabolism.

Effect of Drugs and Medications on PCE Metabolism

Several commonly used drugs affect the pharmacokinetics of TCE metabolism and are also likely to affect the metabolism of PCE (Rouhou et al. 2013; Rouhou et al. 2015). Both Naproxen and salicylic acid (aspirin) increase the metabolism of TCE by rat hepatocytes through the oxidative pathway to TCA and trichloroethanol (TCOH). In contrast, Acetaminophen, Cimetidine, Diclofenac, Gliclazide and Valproic acid decrease the oxidative metabolism of TCE (Rouhou et al. 2013; Rouhou et al. 2015). Naproxen and salicylic acid are commonly used non-

steroidal antiinflamatory drugs. Valproic acid and Cimetidine are commonly used anticonvulsives, and Gliclazide is an anti-diabetic. Phenobarbital induces CYPs which in turn increases the oxidative metabolism of PCE in Long-Evans rats (Costa and Ivanetich 1980). Both phenobarbitol and ethanol enhance the toxicity of TCE (Nakajima et al. 1988). Dexamethazone induces CYP3A and increases conversion of TCVC into TCVC sulfoxide by over 10-times in Wistar rats (Werner et al. 1996). Levels of GSTT1 in various tissues are increased by acetic salicylic acid, alpha-tocopherol, coumarin, phenabarbitol and indole-3-carbinol (Landi 2000). Drugs, steroids, and other compounds that are substrates or inhibitors of MRP2/OAT1/3 transporters also have the potential for inhibiting these transporters and causing drug-toxicant interactions (Vallon et al. 2008).

Several of these drugs are often taken chronically. If co-exposures occur, the internal doses of TCE/PCE and their metabolites could be altered, and therefore increase risk to the population taking these medications (Rouhou et al. 2015). Co-exposures to drugs that inhibit the oxidative metabolism of PCE could also result in more flux through the GSH conjugation pathway.

Nutritional Effects on Cancers and GSTs

Several studies have found that certain diets, including those rich in cruciferous vegetables can reduce cancer risks in humans. High consumption of cruciferous vegetables has been associated with reduced kidney cancer risk (Moore et al. 2007). Consumption of cruciferous vegetables/juices induces GSTs and other enzymes that help reduce risks of breast, bladder, colon and testicular cancers (Steinkellner et al. 2001). Levels of GST theta are enhanced by the ingestion of cruciferous vegetables in *GSTT1*-positive humans (Landi 2000). Other studies have

shown that several GSTs are induced by sulforaphane, an isothiocyanate compound, and by alpha-Lipoic Acid, an antioxidant, that are rich in cruciferous vegetables (Lii et al. 2010).

Dietary and other effects that increase GSH and GST activity likely reduces oxidative stress and augment detoxification of several chemicals, including PAH epoxides, thereby reducing the incidence of disease from such environmental contaminants (Steinkellner et al. 2001; Wang et al. 2004). However, studies in rat kidney cells show that increasing GSH levels increases the toxicity of TCE and PCE (Lash et al. 2007). Thus for individuals exposed to TCE or PCE, rather than promoting health, diet-related increases in GSH and GST activity may increase flux through the GSH conjugation pathway and production of reactive/toxic/mutagenic metabolites.

Potential range of individual variation in bioactivation in the GSH conjugation pathway of PCE/TCE metabolism

The potential range of individual variation in bioactivation through the GSH conjugation pathway of PCE metabolism in humans was estimated using data reported in the literature for each enzyme or step in this pathway. We estimate that individuals may potentially show up to about a 1000-fold range in production of the reactive DCTK through the GSH conjugation/β-lyase pathway of PCE metabolism. This estimate is based on the range of individual variation in DCVG formation observed between 20 human livers (Lash et al. 1999a), a portion of the normal range of variation in GGT (Whitfield 2001), and the range of individual variation in the ratio of overall flux through β-lyase versus through N-Acetylation for a similar substrate between 20 human kidneys (Gul Altuntas and Kharasch 2002). We estimate that individuals may potentially show up to about an 800-fold range in production of TCVC sulfoxide and NAcTCVC sulfoxide

through the GSH conjugation pathway of PCE metabolism. This estimate is based on the range of individual variation in DCVG formation, in GGT activity (Lash et al. 1999a; Whitfield 2001), and in CYP3A and FMO3 activities (Cashman and Zhang 2002; Lamba et al. 2002; Keshava et al. 2004; Nagashima et al. 2009). However, the actual range of variation between individuals in bioactivation through the GSH conjugation pathway might be considerably larger if other sources of variation are also considered, including variation due to transporters, genetic/ethnic diversity, age, gender, diet and pharmaceutical co-exposures.

CONCLUSIONS AND RECOMMENDATIONS

A biochemical and genetic approach is used to better understand the potential contribution of population variability to the 3000-fold range of modeled estimates for GSH conjugation of PCE in humans estimated by Chiu and Ginsberg (2011). The approach examined the genetic, ethnic and other sources of variation in the genes/enzymes controlling the GSH conjugation pathway and their association with chlorinated solvents. The data show that several genes coding for enzymes in the pathway, including *GSTT1, GSTM1*, and *CCBL1* are associated with renal cell cancer (RCC) risk in chlorinated solvent/TCE exposed workers. Population genetic data show diverse ethnic populations differ at these loci and suggest that some ethnic populations are likely more susceptible to chlorinated solvent/TCE-induced RCC than the European workers examined in the original studies. Genetic polymorphisms are also common at several other loci, including *AGXT2, NAT8, ACY3, MRP2, OAT1/3, FMO3* and *CYP3A* that code for enzymes or transporters involved in the GSH conjugation pathway of TCE and PCE metabolism. While effects of these loci on PCE metabolism/toxicity has yet to be examined, several of these genetic polymorphisms

differ between ethnic populations and have been shown to result in altered enzyme activities/deficiencies, altered function and/or disease risk. The high level of genetic diversity in *GSTT1, GSTM1, CCBL1* and several other loci involved in PCE metabolism between ethnic populations is likely to influence levels of toxic and mutagenic metabolites produced through the GSH conjugation/β-lyase pathway. The literature shows that age, gender, diet, and common pharmaceutical co-exposures influence enzymes in the GSH conjugation/β-lyase pathway of PCE metabolism. In addition, several metabolites generated by the GSH conjugation/β-lyase pathway of PCE metabolism including TCVT, DCTK, TCVC sulfoxide and *N*-acetyl trichlorovinyl cysteine (NAcTCVC) sulfoxide are generally considered to be more toxic and/or mutagenic than those produced by the oxidative pathway (Lash et al. 1998a; Lash and Parker 2001; Irving and Elfarra 2013).

Given the diverse ethnic populations in the United States and the variability in PCE metabolism due to genetics, age, gender, diet, and common pharmaceutical co-exposures, large differences in the GSH conjugation/β-lyase pathway of PCE metabolism are expected. As discussed in *Science and Decisions: Advancing Risk Assessment* (NRC 2009) and in Zeise et al. (2013), current cancer risk assessments lack the impact that human inter individual variability such as genetics, may contribute to increased human susceptibility from exposure to environmental pollutants like PCE. Until additional information on the GSH conjugation pathway of PCE metabolism is available, the risk assessment for PCE should consider total metabolism of PCE, including oxidative metabolism and the GSH conjugation/β-lyase pathway.

This review also identified several genes that are likely candidates controlling variation in toxicokinetic and toxicodynamic responses to TCE and PCE. Since genetic variation controls a portion of the phenotypic variability in susceptibility to these contaminants, future human toxicological and epidemiological studies on TCE/PCE should also determine and associate candidate gene SNPs/genotypes with response endpoints. Accounting for genetic differences in susceptibility to TCE/PCE is likely to improve the characterization of genetically susceptible populations, reduce the variability in health risk estimates due to genetic variation and better ensure that toxicity criteria used in human health risk assessments are health protective for diverse ethnic populations.

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Table 1: Estimated frequency of high TCE-induced RCC risk GST and CCBL1 genotypes in diverse ethnic populations.

HapMap Population*	Percent GSTM1 Active ** +/+ or +/-	Percent GSTM1 Null -/-	Percent GSTT1 Active +/+ or +/-	Percent GSTT1 Null -/-	Percent likely Active at both GSTT1 and GSTM1 ***	Percent with High RCC Risk allele at CCBL1****	Percent with Low RCC Risk allele at CCBL1****	Percent with high TCE-induced RCC Risk alleles at GSTT1 and at CCBL1
ASW (A)	78	22	79	21	62	79	21	63
CEU (C)	41	59	89	11	36	38	62	34
CHB (H)	52	48	55	45	29	42	58	23
CHD (D)	44	56	60	40	26	52	48	31
GIH (G)	68	32	80	20	55	60	40	48
JPT (J)	49	51	60	40	30	40	60	24
LWK (L)	77	23	70	30	54	85	16	59
MEX (M)	53	47	78	22	42	41	59	32
MKK (K)	73	27	65	35	48	85	15	55
TSI (T)	45	55	82	18	37	51	49	42
YRI (Y)	80	20	66	34	53	96	4	64
					Corresponds to Highest risk in Bruning et al., (1997).	High B-Lyase RefSnp Rs2280841	Low B-Lyase RefSnp Rs2280841	Corresponds to highest risk in Moore et al., (2010).

*International Haplotype Map (HapMap) Population descriptors:

ASW (A): African ancestry in Southwest USA

LWK (L): Luhya in Webuye, Kenya

CEU (C): Utah residents with Northern/Western European ancestry

MEX (M): Mexican ancestry in Los Angeles, CA

CHB (H): Han Chinese in Beijing, China

MKK (K): Maasai in Kinyawa, Kenya

CHD (D): Chinese in Metropolitan Denver, Colorado

TSI (T): Tuscan in Italy

GIH (G): Gujarati Indians in Houston, Texas

YRI (Y): Yoruban in Ibadan, Nigeria

JPT (J): Japanese in Tokyo, Japan

** Data on GSTM1 and GSTT1 frequency in HapMap populations are from Karaca, et al., (2015). Data are presented as percent of individuals with one or more active GST alleles in order to estimate percentage of individuals with Renal Cell Cancer (RCC)-susceptible genotypes within each ethnic group based on the studies of Brüning et al., (1997) and Moore et al., (2010).

*** Positive alleles at GSTT1 and GSTM1 associated with Renal Cell Cancer (RCC) risk in TCE-exposed individuals (Brüning et al., 1997).

**** GSTT1 and CCBL1 RefSnp rs2280841 associated with RCC risk in TCE exposed individuals (Moore et al., 2010).

Table 2. Gene Frequency of Single Nucleotide Polymorphisms in HapMap populations at Candidate Genes Coding for Enzymes in the GSH Conjugation/B-Lyase Pathway of TCE/PCE Metabolism

Enzyme	L-Alanine-glyoxylate Aminotransferase		Cysteinyl S-Conjugate N-Acetyltransferase		Cysteinyl S-Conjugate N-Acetyltransferase		Aminoacylase III ***	
Locus	*AGXT2*	*AGXT2*	*NAT8*	*NAT8*	*NAT8*	*NAT8*	*ACY3*	*ACY3*
RefSnp	rs37369	rs37369	rs4852954	rs4852954	rs13538	rs13538	rs948445	rs948445
	C	**T**	**C**	**T**	**C**	**T**	**C**	**T**
**HapMap Population	****	****						
ASW (A)	**0.509**	**0.491**	**0.763**	**0.237**	*		**0.472**	**0.528**
CEU (C)	**0.912**	**0.088**	**0.381**	**0.619**	**0.208**	**0.792**	**0.167**	**0.833**
CHB (H)	**0.416**	**0.584**	**0.321**	**0.679**			**0.196**	**0.804**
CHD (D)	**0.472**	**0.528**	**0.317**	**0.683**			**0.222**	**0.778**
GIH (G)	**0.738**	**0.262**	**0.292**	**0.708**			**0.05**	**0.95**
JPT (J)	**0.327**	**0.673**	**0.35**	**0.65**	**0**	**1**	**0.272**	**0.728**
LWK (L)	**0.355**	**0.645**	**0.877**	**0.123**				
MEX (M)	**0.698**	**0.302**	**0.298**	**0.702**			**0.079**	**0.921**
MKK (K)	**0.548**	**0.452**	**0.675**	**0.325**			**0.481**	**0.519**
TSI (T)	**0.902**	**0.098**	**0.402**	**0.598**			**0.176**	**0.824**
YRI (Y)	**0.333**	**0.667**	**0.85**	**0.15**	**0.568**	**0.432**	**0.604**	**0.396**

*Data is not available for empty cells. Note that if a population is in Hardy-Weinberg equilibrium, expected genotype frequencies are P^2, 2PQ and Q^2 for the first allele homozygote, heterozygote, and second allele homozygote, respectively.

**See Table 1 for International Haplotype Map (HapMap) Population Descriptors.

***In regard to refSNP alleles in dbSNP b126 for Human HapMap populations: *AGXT2* rs373689 is associated with hyper-β-aminoisobutyric aciduria (Suhre et al., 2011). *NAT8* rs13538 is associated with metabolites of this enzyme, eGFR and chronic kidney disease (Yu et al., 2014). *ACY3* rs2514036 and rs948445 are associated with blood pressure response to

Bisoprolol (Hiltunen et al., 2015). *ACY3* rs948445 is a missence splice acceptor variant 2kb upstream.

[****]Alleles such as those shown in yellow are anticipated to decrease, while alleles such as those shown in red are anticipated to increase production of toxic/mutagenic metabolites in PCE/TCE exposed individuals.

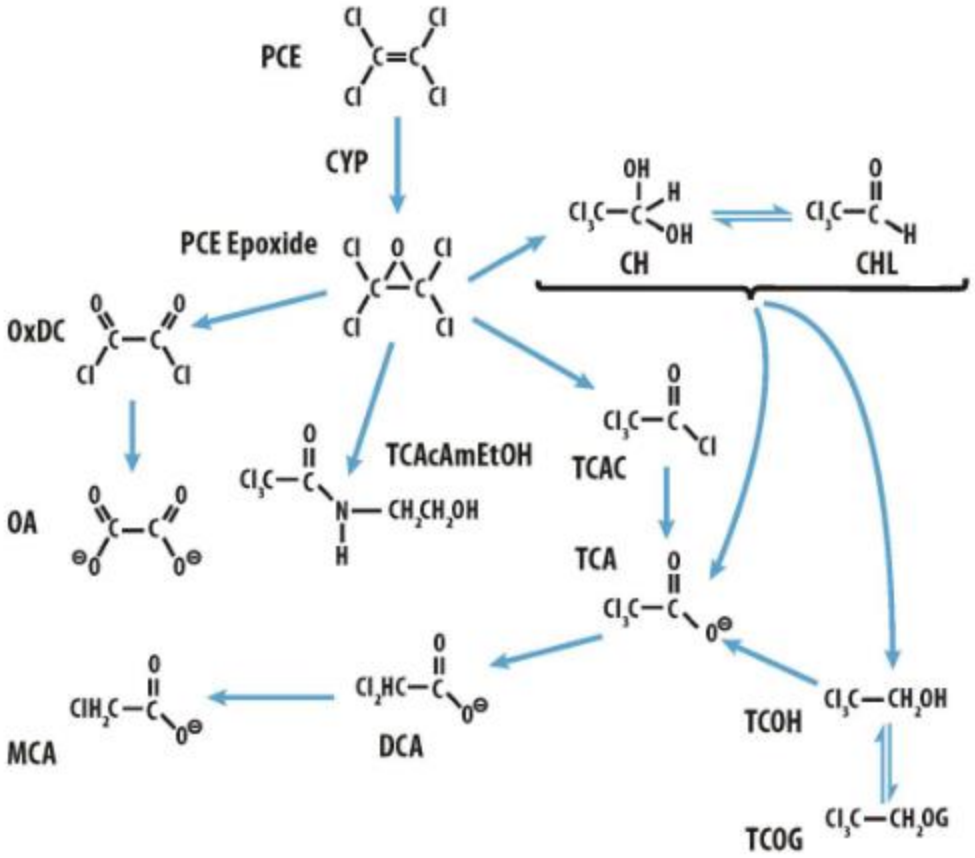

Figure 1. Postulated pathways of Perchloroethylene (PCE) oxidative metabolism. PCE is oxidized by cytochrome P450 (CYP) to form the PCE epoxide intermediate, which can be converted to other metabolites, including chloral hydrate (CH), chloral (CHL), trichloroacetyl chloride (TCAC), oxalate dichloride (OxDC), or trichloroacetyl aminoethanol (TCAcAmEtOH). Trichloroacetate (TCA) can be produced from either TCAC, CH, CHL, or trichloroethanol (TCOH) and its glucuronide (TCOG). TCA can be dechlorinated to form dichloroacetate (DCA) and then monochloroacetate (MCA). OxDC can be dechlorinated to form oxalate (OA).

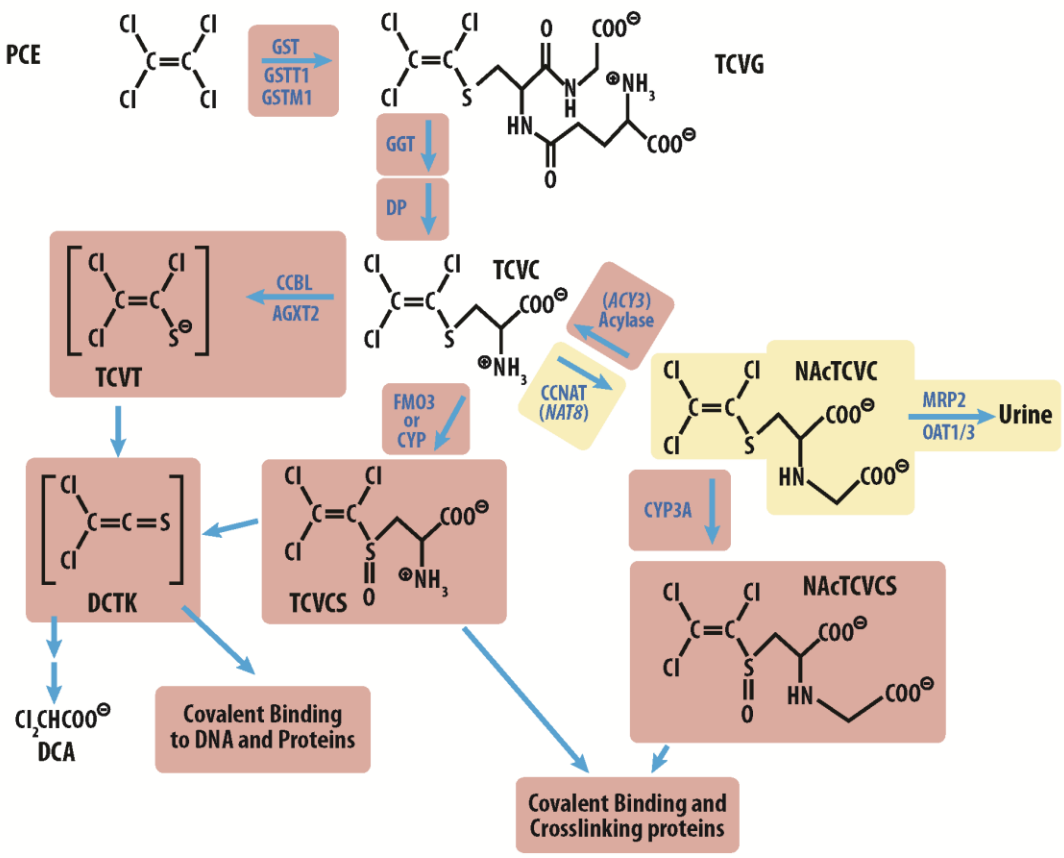

Figure 2. Postulated pathways of Perchloroethylene (PCE) glutathione-dependent metabolism. PCE is conjugated with glutathione (GSH) by glutathione-S-transferase (GST), including isozymes GSTT1 and GSTM1 to form trichlorovinyl glutathione (TCVG). γ-glutamyl transpeptidase (GGT) and cysteinylglycine dipeptidase (DP) then produce *S*-trichlorovinyl-l-cysteine (TCVC). From this branch point, TCVC can be activated by cystene conjugate β-lyase (CCBL) or alanine-glyoxylate amino transferase (*AGXT2*) to form the reactive species, trichlorovinylthiol (TCVT) and dichlorothioketene (DCTK). TCVC may be acetylated by cysteine conjugate *N*-acetyltransferase (CCNAT) to form *N*-acetyl trichlorovinyl cysteine

(NAcTCVC) which can be transported into the urine by multi drug resistance protein 2 (MRP2) and organic ion transporter (OAT) 1/3. Acylase can deacylate NaAcTCVC, converting it back to TCVC. TCVC can also be activated by flavin-containing monooxygenases (FMO3s) or CYP3A to form TCVC sulfoxide. CYP3A can also activate NaAcTCVC to a toxic sulfoxide. Reactive compounds are shown in brackets. DCTK can covalently bind DNA and proteins. TCVC sulfoxide and NaAcTCVC sulfoxide can covalently bind and crosslink proteins. Shows enzymes leading to production of toxic metabolites and the resulting toxic/reactive metabolites. Shows enzymes responsible for detoxification and the resulting non-toxic metabolites.