GENETIC POLYMORPHISMS
IN DISEASE SUSCEPTIBILITY:
GENE-GENE AND GENE-ENVIRONMENT INTERACTIONS

By

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Submitted to the graduate degree program of Toxicology
and the Faculty of the Graduate School of the University of Kansas
In partial fulfillment of the requirements for the degree of
Master's of Science

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Date Defended: __April 26, 2007____

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That this is the approved Version of the following thesis:

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ABSTRACT

Genetic polymorphisms within genes encoding biotransformation enzymes can alter the biotransformation process of exogenous and endogenous chemicals. The purpose of this thesis is to review and evaluate the associations between genetic polymorphisms of biotransformation enzymes, including microsomal epoxide hydrolase (mEH), NAD(P)H quinone oxidoreductase (NQO1), glutathione S-transferase (GST) mu 1 (GSTM1), GST theta 1 (GSTT1), and GST pi 1 (GSTP1), and the transcription factor, nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2), and diseases of the liver, bladder, and lung, and Parkinson's disease (PD). This evaluation will provide an analysis of the overall associations between the polymorphisms and disease and how and these associations are dependent on gene-gene and gene-environment interactions.

Overall, genetic polymorphisms within the biotransformation enzymes evaluated in this thesis, alone, are unlikely to be significant susceptibility or protective factors in the development of disease. Rather, their role as susceptibility or protective factors ultimately depends on gene-gene and gene-environment interactions. Also, dose, length of exposure, the type of xenobiotic, diet, and other factors can be the difference between a polymorphism being a susceptibility or protective factor. Given this differential susceptibility, associations between polymorphisms and disease risk will have to be evaluated on a chemical-specific and mechanistic basis.

To my Wife

ACKNOWLEDGEMENTS

I greatly thank my advisor, Dr. Curtis D. Klaassen, for providing me an opportunity to study Toxicology at the University of Kansas. It was a tremendous honor to be his student. In addition to his guidance and support, his commitment to my learning experience and research has been rewarding and made me a better scientist and person.

I also thank the members of my committee, Drs. Thomas Pazdernik, Yvonne Wan, and Xiaobo Zhong. Their encouragement, guidance, and interest in my research greatly enhanced my learning experience. I also appreciated their suggestions and input on improving my thesis.

I would also like to thank the faculty of the Department for my learning experience. Drs. Karl Rozman and Gregory Reed contributed greatly to my understanding of toxicology, which is reflected in my research. Rosa Meagher was also of great support and assistance throughout my time at KU.

Additionally, I thank EPA and Michael Beringer. EPA provided me the opportunity to pursue a career in toxicology and risk assessment. Michael Beringer's advice and support during my research were also of tremendous help.

Special thanks also go to my friends and family. Their support and encouragement were necessary ingredients in the completion of my studies. I thank Kristian and Martha Fried and David Garrett for their friendship and laughs. I also thank my parents, David and Catherine Johnson, for their support and encouragement.

Finally, I thank my wife, Vicky. I could not have done this without her. She provided me the love, support, and encouragement that I needed to complete one of the most challenging and rewarding experiences of my life.

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LIST OF ABBREVIATIONS

A	Adenine
AFB1	Aflatoxin B1
ala	Alanine
ARE	Antioxidant Response Element
arg	Arginine
BPDE	Benzo(a)pyrene diolepoxide
COPD	Chronic Obstructive Pulmonary Disease
CYP	Cytochrome P450
exo-AFBO	Aflatoxin B1-8,9-exo-epoxide
C	Cytosine
CI	Confidence Interval
G	Guanine
GST	Glutathione *S*-Transferase
GSTM1	Glutathione *S*-Transferase mu 1
GSTT1	Glutathione *S*-Transferase theta 1
GSTP1	Glutathione *S*-Transferease pi 1
HBV	Hepatitis B Virus
HBVAg	Hepatitis B Virus Antigen
HCV	Hepatitis C Virus
HCC	Hepatocellular carcinoma
his	Histidine

IARC	International Agency for Research on Cancer
ile	Isoleucine
MAPEG	Membrane associated proteins in eicosanoid and glutathione
MAO B	Monoamine oxidase B
mEH	Microsomal Epoxide Hydrolase
MnSOD	Manganese-containing superoxide dismutase
NAT1	*N*-acetyltransferase 1
NAT2	*N*-acetyltransferase 2
NQO1	NAD(P)H Quinone Oxidoreductase 1
Nrf2	Nuclear Factor (erythroid-derived 2)-related factor 2
1-OHP	1-hydroxypyrene
OR	Odds Ratio
OTA	Ochratoxin A
PAH	Polycyclic aromatic hydrocarbon
PD	Parkinson's disease
ROS	Reactive Oxygen Species
SCC	Squamous Cell Carcinoma
SNP	Single Nucleotide Polymorphism
T	Thymine
tyr	Tyrosine
UGT	UDP-glucuronosyltransferase

UK	United Kingdom
U.S.	United States
U.S. EPA	United States Environmental Protection Agency
UTR	Untranslated Region
val	Valine
XRCC1	X-ray repair cross-complementing group 1
XRE	Xenobiotic Response Element

Chapter 1: Introduction

1.1 Background

In the field of risk assessment, more specifically dose-response assessment, human variability and uncertainty are important factors to consider when developing toxicity values (e.g., slope factors, reference concentrations) and establishing "safe" exposure levels. In the federal government, dose-response assessments have traditionally relied on default "safety" factors to account for human variability and uncertainty. In recent years, concurrent with the explosion in genetics and genomics technologies, federal agencies such as the United States Environmental Protection Agency (U.S. EPA) have begun to move away from the across-the-board use of default values. Documents such as the U.S. EPA's Guidelines for Carcinogen Risk Assessment (U.S.EPA, 2005) have directed future assessments to focus more on differences in susceptibility to account for human variability and derive uncertainty factors.

Numerous toxicokinetic factors can contribute to the variability in the response to a chemical insult whether they include increases or decreases in excretion, alterations in plasma protein levels that affect the distribution of xenobiotics to target organs, or an increase or decrease in absorption of a chemical. Notwithstanding these other toxicokinetic principles, genetic polymorphisms within genes encoding biotransformation enzymes, transporters, transcription factors, DNA-repair enzymes, and other proteins are of considerable interest given the dramatic increase in polymorphism-related research. Genetic polymorphisms within genes

encoding biotransformation enzymes can have numerous impacts that can alter the biotransformation process of exogenous and endogenous chemicals. While many polymorphisms may have no affect at all, polymorphisms can impact the overall function, activity, and stability of an enzyme. Additionally, polymorphisms in the regulatory region of the gene may impact gene expression or mRNA stability (Gentry *et al.*, 2002). As a result of these polymorphisms, individuals may have an increased or impaired ability to detoxify harmful exogenous and endogenous compounds. Also, depending on the substrate, such polymorphisms could also lead to greater rate of bioactivation.

To date numerous studies have been conducted linking genetic polymorphisms to increases and decreases in risk of cancer and other diseases, as well as responses to drugs and chemotherapeutics. In regards to genetic polymorphisms within biotransformation enzymes, such as cytochrome P450s (CYPs), glutathione *S*-transferases (GSTs), microsomal epoxide hydrolase (mEH), *N*-acetyltransferases (NATs), UDP-glucuronosyltransferases (UGTs), NAD(P)H quinone oxidoreductase 1 (NQO1), etc., individual studies have found that linking individual genotypes to disease is not a simple comparison, but rather a complex association involving gene-environment and gene-gene interactions. Of course these same interactions apply to polymorphisms within genes encoding transporters, channels, DNA-repair enzymes, and transcription factors, antioxidants, etc.

Gene-gene interactions are of considerable importance when linking susceptible genotypes to disease. Although there are numerous types of gene-gene

interactions, two gene-gene interactions of interest include interactions between metabolic enzymes with other metabolic enzymes, and interactions between metabolic enzymes, DNA-repair enzymes, and antioxidants. These interactions can be direct or indirect. For example, gene-gene interactions between biotransformation enzymes include interactions where multiple enzymes or isoenzymes compete with each other for a given compound or detoxify or bioactivate the compound at different steps of a compound's biotransformation pathway (Wormhoudt *et al.*, 1999).

Gene-environment interactions are also important and have been broadly defined as the interaction between susceptible genetic factors and environmental factors including infectious, chemical, physical, nutritional, and behavior factors (CDC, 2000). Numerous diseases, such as hepatitis, can influence a person's susceptibility to other diseases (e.g., cancer) through inflammatory processes that generate reactive oxygen species (ROS). Exposures to chemicals, whether they be environmental pollutants or through the diet, can also have the potential to impact associations between genotypes and disease. Chemicals in the environment and diet can induce or inhibit the expression of biotransformation enzymes. For example, tocotrienols, a group of molecules falling within the vitamin E family having antioxidant activity and non-antioxidant activity, have been shown to inhibit glutathione *S*-transferase pi 1 (GSTP1) (Van Haaften *et al.*, 2001; Schaffer *et al.*, 2005). Dietary antioxidants may also offset impaired detoxification pathways. Furthermore, given the body has numerous detoxification pathways and many enzymes share common substrates, the level of exposure to a chemical that a person

is exposed to may determine whether a particular genotype is a susceptibility factor for a given individual. Primary biotransformation pathways may become saturated and reliant on other pathways. Also, the concentration of a particular compound may determine the detoxification pathway.

Another set of interactions, which may fall into both gene-gene and gene-environment interactions, or neither, include interactions involving gender, ethnicity, age, and disease. Differences between the genders and age groups in regards to xenobiotic intake, distribution, excretion, exposure, gene-expression, hormones, and etc. could impact the association between disease and susceptible genotypes. Ethnicity may also impact these associations at the genetic level and at the environment level. At the environment level, dietary patterns may differ, which may include greater or less exposure to dietary antioxidants. Finally, disease histology may modify associations whereby xenobiotics may only be distributed to particular tissues of an organ.

1.2 Statement of Purpose

The purpose of this thesis is to review and evaluate the associations between genetic polymorphisms of the biotransformation enzymes, including mEH, GST mu 1, GST theta 1, GST pi 1, and NQO1 and the transcription factor, nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2), and diseases of the liver, bladder, and lung, and Parkinson's disease (PD). This evaluation will provide an analysis of the overall associations between mEH, GSTM1, GSTT1, GSTP1, NQO1, and Nrf2 genetic polymorphisms and disease, and how and to what extent these associations

are dependent on gene-gene and gene-environment interactions. This evaluation will also include a discussion on potential challenges, implications, and use of such information in regulatory toxicology and medicine.

Note, while comprehensive, this review and evaluation covers a relatively small group of group of genetic polymorphisms and disease, and is not intended to represent the numerous other associations found between polymorphisms of other biotransformation genes, transporters, transcription factors, etc., and risk of disease. However, many of the general principles regarding associations between genetic polymorphisms and disease (e.g., gene-gene and gene-environment interactions) are applicable to other associations not evaluated in this thesis.

The biotransformation enzymes, mEH, GSTM1, GSTT1, and GSTP1, NQO1, and Nrf2 were chosen in that they are all primarily involved in the detoxification of endogenous and exogenous reactive chemicals and intermediates. Also, mEH, GSTM1, GSTT1, GSTP1, and NQO1 share many of the same types of substrates, which may provide insight on gene-gene interactions. For example, both GST and mEH can catalyze the detoxification of reactive epoxides resulting from polyaromatic hydrocarbon (PAH) oxidation, and NQO1 and GSTs both detoxify products of oxidative stress and are controlled in part by the transcription factor, Nrf2.

Additionally, although numerous diseases have been studied with respect to genetic polymorphisms, this thesis primarily focuses on diseases of the lung, liver, and bladder, as well as Parkinson's disease (PD). The lung, liver, and bladder were chosen based on a combination of available research and their physiologic location

and role (i.e., "first-pass" metabolism and/or excretion). Although the etiology of PD is relatively unknown, the disease is characterized by degeneration of nigrostriatal dopaminergic neurons (Simonian and Coyle, 1996), where it is hypothesized that oxidative stress plays a critical role in the development of this disease (Olanow and Tatton, 1999). This is supported in studies showing increase indices of oxidative stress, such as increased iron levels, increased lipid peroxidation, and decreased levels of glutathione (Hirsch *et al.*, 1991; Jenner, 1993). Given products of oxidative stress are substrates for mEH, GSTs, and NQO1, this disease was chosen for evaluation. Precursors of oxidative stress that may lead to PD may include endogenous and exogenous factors such as *o*-quinones of catecholamines and neurotoxic pesticides (Tanner, 1989; Baez *et al.*, 1997; Ascherio *et al.*, 2006; Brown *et al.*, 2006).

Chapter 2: Enzymology

2.1 Introduction

As noted previously mEH, GSTM1, GSTT1, and GSTP1, NQO1, and Nrf2 are all primarily involved in the detoxification of endogenous and exogenous reactive chemicals and intermediates. These enzymes also share many of the same types of substrates. The following sections provide a background on the enzymes evaluated in this thesis including function, substrate, and polymorphisms, with special emphasis on the particular polymorphisms that will be evaluated in this thesis.

2.2 Microsomal Epoxide Hydrolase

Microsomal epoxide hydrolase is a phase-I enzyme with broad substrate specificity that is expressed in numerous tissues and cell types. mEH catalyzes the hydrolysis of a large number of alkene and arene epoxides to *trans*-dihydrodiols (Wormhoudt *et al.*, 1999; Fretland and Omiecinski, 2000). Although mEH is primarily involved in the detoxification of reactive epoxide intermediates (Guengerich and Davidson, 1982; Armstrong, 1987), the enzyme does exhibit a dual role in that it also participates in bioactivation pathways for some substances (Hosagrahara *et al.*, 2004). For example, *trans*-dihydrodiols of polycyclic aromatic hydrocarbons (PAHs) can be further activated by P450s catalysis to form highly electrophilic and mutagenic bay region diol-epoxides (Sayer *et al.*, 1985; Shou *et al.*, 1996).

A total of 164 SNPs have been identified within the mEH gene with SNPs being located in the promoter region, exons, introns, exon/intron boundary, and 3'

untranslated region (UTR) (CHIP, 2007). Of particular interest are two SNPs within the gene's coding region, which include SNPs in exon 3 and exon 4. The exon 3 polymorphism results in a tyrosine for histidine substitution at amino acid position 113 (tyr113his), whereas the exon 4 polymorphism results in a histidine for arginine substitution at amino acid position 139 (his139arg) (Gaedigk *et al.*, 1994; Hassett *et al.*, 1994; Hassett *et al.*, 1997). Both variants have shown to moderately affect the overall activity of the enzyme in *vitro* (Hassett *et al.*, 1994; Hassett *et al.*, 1997; Omiecinski *et al.*, 2000). The 113 variant, where tyrosine is replaced with histidine, results in a less active enzyme, whereas the 139 variant results in a more active enzyme. When considering the impacts of combinations of the variants with the non-variants at either position, there are several gradations of predicted activity ranging from very low activity through intermediate activity to high activity (Smith and Harrison, 1997; Benhamou *et al.*, 1998; Kiyohara *et al.*, 2006). Table 1 provides the predicted activities for combinations of the polymorphisms.

Table 1. Predicted mEH Activity

	High Activity	Intermediate Activity	Low Activity	Very Low Activity
Exon 3 his/his Exon 4 his/his				
Exon 3 his/his Exon 4 his/arg				
Exon 3 his/tyr Exon 4 his/his				
Exon 3 his/his Exon 4 arg/arg				
Exon 3 his/tyr Exon 4 his/arg				
Exon 3 his/tyr Exon 4 arg/arg				
Exon 3 tyr/tyr Exon 4 his/his				
Exon 3 tyr/tyr Exon 4 arg/his				
Exon 3 tyr/try Exon 4 arg/arg				

Shaded area represents predicted activity (Benhamou *et al.*, 1998; Smith and Harrison, 1997; Kiyohara *et al.*, 2006)

2.2 NAD(P)H Quinone Oxidoreductase

NQO1 is an inducible enzyme regulated by antioxidant response elements (AREs) and xenobiotic response elements (XREs) (Ross *et al.*, 2000). NQO1 has three functions in the cell. First, it is responsible for two electron reduction of reactive quinones to the hydroquinones, which can be further conjugated and excreted, thereby circumventing single electron reduction and the production of reactive semiquinones that can react with cellular macromolecules or undergo further redox-cycling (Joseph *et al.*, 1994; Nioi and Hayes, 2004). Other substrates include quinone epoxides, quinoneimines, azo dyes, and C-nitroso derivatives of arylamines (Brunmark *et al.*, 1987; Klaassen, 2001). In addition to this role, NQO1 is involved in two other mechanisms of defense, which include maintenance of endogenous antioxidants, such as α-tocopherol-hydroquinone and the stabilization of the reduced form of the p53 tumor suppressor protein (Ross *et al.*, 2000; Nioi and Hayes, 2004).

So far, 93 SNPs have been identified in the NQO1 gene with SNPs being located in the promoter region, introns, exon/intron boundary, 3' UTR, and coding region of the gene including a SNP at base-pair 609 resulting in a cytosine to thymine substitution (Ross *et al.*, 2000; CHIP, 2007). Although the impact of the polymorphisms in other parts of the gene are unknown, the 609 variant resulting in an amino acid change of proline to serine at position 187 results in an unstable enzyme that yields virtually no activity, due to accelerated degradation via the ubiquitin/proteasome system (Siegel *et al.*, 1999; Siegel *et al.*, 2001). The frequency ranges from 4 to 20% and differs by ethnicity (Ross *et al.*, 2000).

2.3. Glutathione *S*-Transferase

Glutathione *S*-transferases constitute a superfamily of dimeric enzymes that have several biological roles including catalyzing the conjugation of glutathione (GSH) to numerous types of endogenous and exogenous electrophilic substrates. These enzymes are upregulated during oxidative stress (Nebert and Vasiliou, 2004), and have been divided into three categories including cytosolic, mitochondrial, and microsomal Membrane Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEG) GSTs (Hayes *et al.*, 2005). In regards to cytosolic GSTs, this group of GSTs is comprised of seven classes, including alpha, mu, pi, sigma, theta, zeta, and omega and within each class of enzymes there are isoforms. For example, GST mu (GSTM) comprises of five mu genes (GSTM1, GSTM2, GSTM3, GSTM4, and GSTM5) located in a 20 kb cluster on chromosome 1p (Xu *et al.*, 1998; Strange *et al.*, 2001). Of interest is that the subunits within Alpha and Mu class families can form heterodimers (Hayes and Pulford, 1995; Hayes *et al.*, 2005).

With regards to this thesis, only the genes encoding GSTM1, GSTT1, and GSTP1 are evaluated. These three GST subunits have been studied extensively and play a critical role in detoxification of numerous exogenous compounds such as PAHs, halogenated solvents, pesticides, environmental pollutants, and pharmaceuticals (Hayes *et al.*, 2005). Carcinogenic chemicals, such as activated metabolites of heterocyclic amines, epoxides of PAHs, and aflatoxins are also detoxified by GSTs (Autrup, 2000; Hayes *et al.*, 2005).

2.3.1 GSTM1

GSTM1 is mainly responsible for detoxifying epoxides of aromatic hydrocarbons (i.e., PAHs), aflatoxins, and products of oxidative stress (Autrup, 2000; Hayes *et al.*, 2005; Ye *et al.*, 2006). GSTM1 is expressed highest in liver, but also expressed in bladder, kidney, lung, and nasal tissue (Autrup, 2000; Gilliland *et al.*, 2004; Ye *et al.*, 2006). Genetic polymorphisms of GSTM1 include a complete gene deletion ("null" genotype), which is evaluated in this thesis, a duplicate of the gene, and 83 SNPs (McLellan *et al.*, 1997; Autrup, 2000; CHIP, 2007). The null genotype yields no protein whereas the duplicate gene yields ultra-rapid enzyme activity (McLellan *et al.*, 1997). The SNPs identified within the GSTM1 gene are located in the promoter region, exons, introns, exon/intron boundary, and 3' untranslated region (UTR) (CHIP, 2007). Of considerable interest is that the homozygous "null" genotype is found in approximately 50% of the general population (Ye *et al.*, 2006). However, African-Americans have shown a lower frequency (30%) for the null genotype (Ye *et al.*, 2006).

2.3.2 GSTT1

GSTT1 is expressed mainly in liver and kidney (Sherratt *et al.*, 1997), and it is responsible for the conjugation of GSH with low-molecular-weight halogenated compounds, such as methyl bromide, methyl chloride, ethylene oxide, 1,2-dibromoethane, 1,3-butadiene, and dichloromethane and epoxide intermediates (Wormhoudt *et al.*, 1999; Strange *et al.*, 2001). In some instances, GSTT1 may play a role as a bioactivator (DeMarini *et al.*, 1997; Sherratt *et al.*, 1998). Similar to

GSTM1, GSTT1 has a genetic polymorphism that results in a complete gene deletion. Like GSTM1, the homozygous null genotype is common and the frequency of the deletion displays interethnic variability ranging from 10-20% in Caucasians and up to 65% in the Asian population (Wormhoudt *et al.*, 1999; Strange *et al.*, 2001). In addition to the null polymorphism, which will be evaluated in this thesis, a total of 79 SNPs have been identified within the GSTT1 gene with SNPs being located in the promoter region, exons, introns, exon/intron boundary, and 3' UTR (CHIP, 2007).

2.3.3 GSTP1

The third and final GST this thesis will evaluate is GSTP1. GSTP1 is expressed widely in epithelial tissues and abundant in the lung, esophagus, placenta, and blood-brain barrier (Carder *et al.*, 1990; Autrup, 2000). In these tissues it is responsible for detoxifying numerous compounds including PAHs, lipid-peroxidation products, and DNA-oxidative products.

A total of 122 SNPs have been identified within the GSTP1 gene with SNPs being located in the promoter region, exons, introns, exon/intron boundary, and 3' UTR (CHIP, 2007). The most heavily studied polymorphism, which is primarily evaluated in this thesis is a substitution of adenine for guanine at position 313 (A313G), resulting in a change of isoleucine to valine at amino acid position 105 (ile105val). The other genetic variant that has been studied is at position 341, where cytosine is replaced with thymine, resulting in a change of alanine for valine at amino acid position 114 (ala114val). Both mutant alleles have altered specific activity and affinity for electrophilic substrates resulting in a variant that is several times more

active toward some substrates (e.g., diol epoxides of PAHs) and less active toward others (e.g., 1-chloro-2,4-dinitrobenzene) (Hu and Singh, 1997; Hu *et al.*, 1998; Watson *et al.*, 1998; Strange *et al.*, 2000). Furthermore, the val105 polymorphism may affect enzyme thermal stability (Johansson *et al.*, 1998).

2.4 Nrf2

Nrf2 is a basic leucine zipper transcription factor that is sequestered in the cytoplasm by the kelch-like ECH-associated protein 1 (Keap1). When cells are exposed to oxidative stress and xenobiotics, antioxidant-response element (ARE) activation signals disrupt the Nrf2-Keap1 complex (Lee *et al.*, 2005a). Following dissociation, Nrf2 translocates to the nucleus and in the nucleus it heterodimerizes with other transcription factors (e.g., Maf protein) and binds to the AREs present in phase-II gene promoters (Itoh *et al.*, 1997). Nrf2 and AREs are known to regulate the expression of detoxification genes (e.g., NQO1 and GST), as well as antioxidant genes, NAD(P)H, UGTs, and glutathione biosynthesis genes (Venugopal and Jaiswal, 1996; Itoh *et al.*, 1997; Kwak *et al.*, 2001).

In humans, 205 SNPs and one triplet repeat polymorphism have been found in the Nrf2 gene (Yamamoto *et al.*, 2004; CHIP, 2007). SNPs are located in the promoter region, exons, introns, and 3' UTR. The triplet repeat polymorphism is located in the regulatory region of the gene (Yamamoto *et al.*, 2004). To date the impacts of these polymorphisms on the function and expression of the Nrf2 transcription factor are not known, however, impaired function of the transcription factor could have profound effects as observed in numerous studies on mice (Hayes *et*

al., 2000; Chan *et al.*, 2001; Kwak *et al.*, 2001; Cho *et al.*, 2002; McMahon *et al.*, 2003; Lee *et al.*, 2005a; Ma *et al.*, 2006).

Chapter 3: Microsomal Epoxide Hydrolase

3.1 Introduction

This chapter will evaluate the impact of polymorphisms in the gene encoding mEH on risk of diseases of the lung and liver and Parkinson's disease. As will be shown associations between mEH and disease are dependent on gene-gene and gene-environment interactions. This section will also show that the types of associations between mEH and risk of disease (i.e., risk factor, protective factor or neither) are specific to particular target tissues and disease. Furthermore, this chapter will show that the genotype can be both a risk factor and protective factor depending on the disease being evaluated.

3.2 Lung

Microsomal epoxide hydrolase has a significant role in the lung, such as hydrolyzing epoxides of environmental pollutants and constituents of tobacco smoke. Additionally, mEH plays a significant role in detoxifying reactive intermediates of oxidative stress. Given this role, the activity of mEH could significantly impact the overall risk of developing diseases of the lung. As will be shown in this section, the mEH genotype is associated with diseases of the lung and that these associations are dependent on gene-gene and gene-environment interactions.

3.2.1 Lung Cancer

Based on available information, data supports that the mEH genotype is modestly associated with lung cancer risk. However, the type of association, whether it be an increase or decrease in risk, is dependent on environmental exposures,

especially tobacco smoking. Additionally, the association between mEH and lung cancer risk is significantly influenced by gene-gene interactions and may be also be dependent on the combination of exon 3 and exon 4 SNPs within the gene. Other factors to be considered include ethnicity, whether it be a genetic or an environmental component (e.g., diet), and target tissue (i.e., cancer histology).

3.2.1.1 Environmental Interactions

Overall, without consideration of smoking status, a majority of the available research results shows little or no association between mEH genotype and lung cancer risk. At best, a decrease in lung cancer was observed among Caucasians carrying the variant exon 3 allele (low activity) in a recent meta-analysis (Odds Ratio (OR) = 0.65; 95% CI = 0.44-0.96) (Kiyohara *et al.*, 2006). Of interest is this decrease is consistent with the overall associations between the mEH genotype in smokers. The same study found no significant associations between the exon 4 genotypes and risk of lung cancer. Despite these findings, when smoking status is taken into account, significant associations are observed. Additionally, smoking status determines whether mEH genotype is associated with an increase or decrease in cancer risk.

Products of mEH-mediated hydrolysis of PAHs include precursors of ultimate carcinogens, such as benzo(a)pyrene dihydrodiol (Klaassen, 2001). Given this pathway, the higher activity mEH genotypes may lead to a greater production of precursors to ultimate carcinogens and an increase in cancer risk (Benhamou *et al.*, 1998). Likewise, the low activity mEH genotypes may be associated with a decrease in lung cancer risk among smokers. When considering the individuals SNPs, smokers

whom are exposed to high levels of PAHs carrying the exon 3 variant allele have a decrease in cancer risk compared to carriers of the homozygous wild-type genotype (Benhamou *et al.*, 1998; London *et al.*, 2000a; To-Figueras *et al.*, 2001; Gsur *et al.*, 2003; Park *et al.*, 2005a; Voho *et al.*, 2006). For example, in a group of mostly smokers (88.8%), Gsur *et al.* (2003) found that homozygous carriers of the mEH exon 3 variants were at a significantly decreased risk of lung cancer compared to carriers of the mEH exon 3 wild-type (OR = 0.38; 95% CI 0.20-075). A dramatic decrease in lung cancer risk was also observed in African-American lung cancer patients in Los Angeles having the exon 3 variant allele (OR =0.08; 95% CI = 0.01-0.62) (London *et al.*, 2000a). Likewise, the exon 4 variant conferring high activity has also been associated with an increase in cancer risk among Caucasian and Asian smokers (Persson *et al.*, 1999; Zhao *et al.*, 2002; Cajas-Salazar *et al.*, 2003).

Despite these findings, many other studies have shown little or no association between the individual genotypes and lung cancer risk among smokers. Two studies have found no association between the exon 3 variant and cancer risk among groups of mostly smokers (Persson *et al.*, 1999; Zhao *et al.*, 2002). Other studies have also found no association between the exon 4 variant and cancer risk among groups of all or mostly smokers (Benhamou *et al.*, 1998; Gsur *et al.*, 2003; Park *et al.*, 2005a). Such findings may be due in part to the overall combination of the two polymorphisms.

As mentioned previously, the combination of the mEH exon 3 and exon 4 polymorphisms may play a significant role in determining the overall activity of

mEH. Consequently, exon 3 or exon 4 variants alone do not necessarily reflect the overall activity or stability of the enzyme. For example, individuals heterozygous for both alleles are predicted to have intermediate enzyme activity (Benhamou *et al.* 1998; Smith and Harrison 1997). When considering both polymorphisms, the predicted high activity and low activity genotypes have been associated with an increase and decrease in lung cancer risk, respectively, among smokers (Benhamou *et al.*, 1998; London *et al.*, 2000a; Cajas-Salazar *et al.*, 2003; Voho *et al.*, 2006). Studies have found that the predicted high activity genotype is associated with significant increases of risk among smokers with ORs ranging from 2.3 (95% CI = 1.2-4.3) to 2.66 (95% CI = 1.33-5.33) (Benhamou *et al.*, 1998; Cajas-Salazar *et al.*, 2003; Park *et al.*, 2005a). Benhamou *et al.* (1998) also found that predicted intermediate activity genotype was associated with a 1.65-fold (95%, CI = 0.95-2.86) increases in lung cancer risk, respectively. Confirming these findings, Voho *et al.* (2006) and London *et al.* (2000a) found that the predicted low activity genotypes have also been associated with a significant decreases in risk with ORs of 0.51 (95% CI = 0.32-0.82) and 0.10 (95% CI = 0.01-0.83), respectively.

Smoking duration and intensity also impact the association between mEH genotype and lung cancer risk among smokers. In short-term smokers, the mEH genotype may not be associated with lung cancer or as discussed later the associations may be similar to non-smokers. While finding no overall associations between mEH genotype and risk of lung cancer, Zhou *et al.* (2001) found that the very-low activity genotypes were associated with a trend of decreasing risk as pack-years increased.

For smokers who smoked less than 25 to 30 pack-years, no significant associations between the very-low activity mEH genotype and risk of lung cancer was observed, though a trend of increasing risk as pack years decreased was observed. In smokers who smoked greater than 25 to 30 pack-years, there was a trend of decreasing risk for both squamous cell carcinoma and adenocarcinoma. Only the trend for squamous cell carcinoma was significant ($p < 0.01$), as shown in Figure 1.

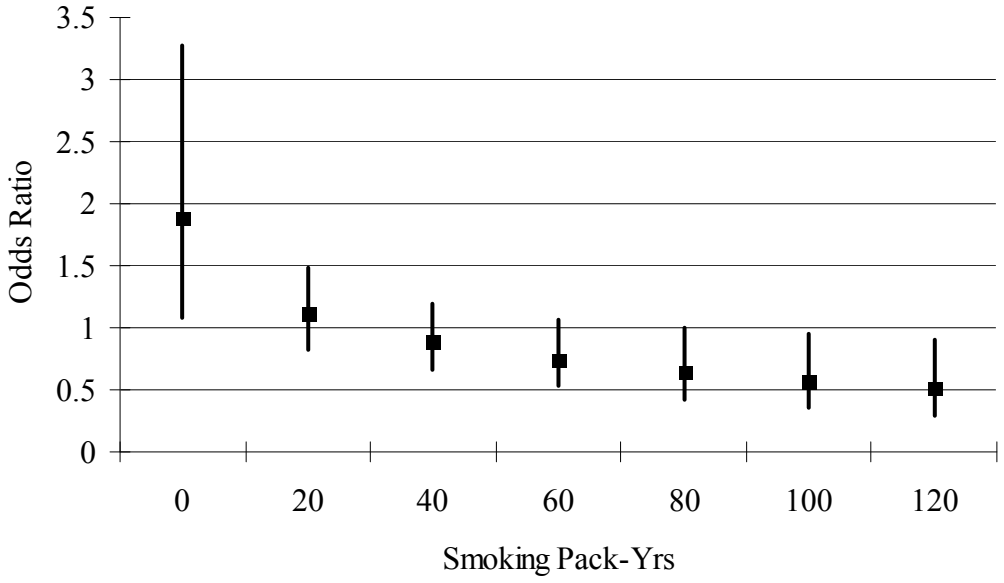

Figure 1. Squamous Cell Carcinoma Risk of Very Low mEH Activity Versus All Other Genotypes ($p < 0.01$). Based on data from Zhou *et al.* (2001)

In support of these findings, the high activity mEH genotype was associated with a significant increase in lung cancer risk (OR = 9.69; 95% CI = 3.31-28.38) for French Caucasians who smoked greater than 30 years (Benhamou *et al.*, 1998). Among the <30 years smoking group, a non-significant increase was observed, but may have been due to the very small sample size ($n = 6$).

Despite the findings by Zhou *et al.* (2001) and Benhamou *et al.* (1998) , Park *et al.* (2005) found that Caucasian light smokers (< 46 pack-years) with intermediate or high-activity genotypes had increase in lung cancer risk, whereas no increase was observed in the > 46 pack-years group. However, the results did not reach statistical significance. In addition, the sample group was quite small.

In support of the role of gene-environment interactions, the mEH genotypes appear to have the opposite effect in non-smokers where the predicted low and high genotypes may be associated with an increase and decrease in lung cancer risk, respectively. In this instance, non-smokers exposed to environmental pollutants have an inability to detoxify reactive epoxide intermediates of environmental pollutants (Zhou *et al.*, 2001). In support of this, Zhou *et al.* (2001) found that the very-low enzymatic activity genotypes compared to all other genotypes were associated with a significant increase in cancer risk among non-smokers (OR = 1.89; 95% CI = 1.08 – 3.28). A significant increase in lung cancer risk was also observed in non-smokers having the very-low activity genotype in Czechs (OR = 11.23; 95% CI = 1.48-88.41) (Habalova *et al.*, 2004). However, these results should be interpreted with caution given the small sample size (n = 4).

In addition to the impacts of smoking and other environmental exposures may have on the association between mEH genotype and lung cancer risk, other factors may impact these associations. Such factors include the cancer histological type, ethnicity, and age. Previous studies have shown that PAHs have been closely related to squamous cell carcinoma, whereas nitrosamines have been related to

adenocarcinoma (Deutsch-Wenzel *et al.*, 1983; Hoffmann *et al.*, 1996; Le Marchand *et al.*, 1998). Furthermore, over the past 50 to 60 years, cigarettes have evolved with the advent of filters and reformulations, thereby reducing PAH exposure and increasing nitrosamine exposure (Wynder and Hoffmann, 1994; Le Marchand *et al.*, 1998). Consequently, there has been a shift in lung cancer histology with adenocarcinoma surpassing squamous cell carcinoma as the most frequent type of lung cancer in developed countries (Travis *et al.*, 1995). Based on these findings, it might be expected that mEH polymorphisms may have significant associations with risk of squamous cell carcinoma, but not adenocarcinoma. However, such findings have been inconsistent, but do show that histological type is an important factor to consider when evaluating the association between mEH and lung cancer risk. For example, as discussed earlier, Zhou *et al.* (2001) found significant associations between squamous cell carcinoma and the mEH genotype, but not among adenocarcinoma cases. Similar results were also observed in a Chinese population where the high/intermediate genotypes were associated with a significant increase in squamous cell carcinoma in a group of smokers and non-smokers (OR = 1.96; 95% CI = 1.04-3.70) (Lin *et al.*, 2000). The percentage of smokers in the squamous cell carcinoma group was not reported. In contrast to these findings, the high mEH activity genotypes were significantly associated with an increase risk of adenocarcinoma, but not squamous cell carcinoma in Caucasian smokers from the U.S. (Park *et al.*, 2005a). Consistent with these findings, Gsur *et al.* ((Gsur *et al.*,

2003) found that Caucasians from Austria carrying the exon 3 variant allele with predicted low activity had a significant decrease in adenocarcinoma.

Ethnicity may too have an effect on the association between mEH genotypes and lung cancer risk. In a meta-analysis by Kiyohara *et al.* (2006), the exon 3 homozygous variant compared to the homozygous wild-type was associated with a slight decrease in lung cancer risk (OR = 0.71; 95% CI = 0.52-0.99) in Caucasians. No significant association was found in the much smaller group of Asians (OR = 1.37; 95% CI = 0.83-2.27). Also, as noted earlier, the exon 3 homozygous variant has been associated with a significant decrease in African-American smokers from Los Angeles (London *et al.*, 2000a). However, these results were not confirmed in another study by Wu *et al.* (2001), but given the mEH exon 3 homozygous variant genotype is rare in African-Americans and the study group was small (n = 78), these results are uncertain. A similar lack of association was also observed in Mexican-Americans carrying the mEH exon 3 variant, but individuals having the exon 4 variant had a statistically significant increase in lung cancer risk with an OR of 3.6 (95% CI = 1.26-10.42).

Available data also suggests that age and cumulative exposures may play a role in the association between mEH genotype and risk of lung cancer. Zhao *et al.* (2002) suggested mEH variants have a significant impact on lung cancer development early in life, whereas cumulative exposures play a more important role later in life. This was supported in their study that found that the high activity genotype in Caucasians less than 64 years of age were at a 4.95-fold (95% CI = 1.65-14.86)

increase risk of lung cancer. Confirming these findings, Wu *et al.* (2001) found that Mexican-Americans carrying the exon 4 variant under 65 years age had an increase in lung cancer risk (OR = 7.4; 95% CI = 1.36-40.23).

3.2.1.2 Gene-Gene Interactions

Gene-gene interactions play a more significant role in whether mEH genotypes play a protective or susceptibility role in diseases of the lung and may explain some of the heterogeneity and/or lack of associations observed in the previously mentioned studies. In fact, such interactions may be more important than environmental factors. In addition, associations between mEH genotype and risk of lung cancer are modified by gene-gene interactions in which the other gene does not code a biotransformation enzyme.

As discussed previously, the mEH highly active genotype is slightly associated with a decrease in lung cancer among smokers and an increase in lung cancer among non-smokers. This slight association may be likely due in part of other enzymatic pathways being able to detoxify reactive diols generated from mEH biotransformation or compensation by other pathway as a result of decreased in ability to detoxify reactive epoxides. However, when these other genotypes yield a less protective enzyme, stronger associations between the mEH genotype and risk of lung cancer are observed. For example, while neither genotype alone reached statistical significance, To-Figueras *et al.* (2001) found that carriers of the homozygous exon 3 wild-type mEH genotype combined with the wild-type (105ile/ile) GSTP1 genotype were associated with a significant increase in lung

cancer risk (OR = 2.34; 95% CI = 1.21- 4.52). No interactions were observed between the mEH exon 4 genotype and GSTP1 genotypes or between mEH and other GSTs including GSTM1 and GSTT1. When evaluating the data according to predicted mEH activity, carriers of the predicted high and intermediate mEH genotypes were only associated with significant increases in lung cancer risk in individuals carrying wild-type (105 ile/ile) GSTP1 genotype. As a result, the study concluded that these individuals having the less active form of GSTP1 are unable to conjugate the mutagenic benzo(a)pyrene diolepoxide (BPDE) resulting from mEH catalysis of the benzo(a)pyrene (Smith and Harrison, 1997; To-Figueras *et al.*, 2001).

The association between mEH and risk of lung disease may also involve associations with P450s, in particular CYP1A1, which primarily catalyzes the metabolism of large polyaromatic hydrocarbons, such as benzo(a)pyrene (Wormhoudt *et al.*, 1999). Several polymorphisms have been found in the gene encoding CYP1A1 that affect the overall activity of the enzyme. When combining the high activity CYP1A1*2A/2A genotype with the mEH high and normal activity genotypes, Lin *et al.* (2000) found a significant increase in squamous cell carcinoma (OR = 6.76; 95% CI = 2.29-19.10), as well as total lung cancer (OR= 2.56; 95% CI = 1.08–6.10) (Figure 2). No associations were found in adenocarcinoma cases. The genotypes alone were also not significantly associated with overall lung cancer, however, the CYP1A1 variant and mEH high/normal activity genotypes were significantly associated with squamous cell carcinoma cases with ORs of 2.86 (95% CI = 1.33–6.12) and 1.96 (95% CI = 1.04–3.70), respectively. Yet, as shown in

Figure 2, the mEH high/normal and CYP1A1 variant activity genotype were not significantly associated with squamous cell carcinoma risk when combined with the CYP1A1 wild-type and mEH low activity genotypes, respectively.

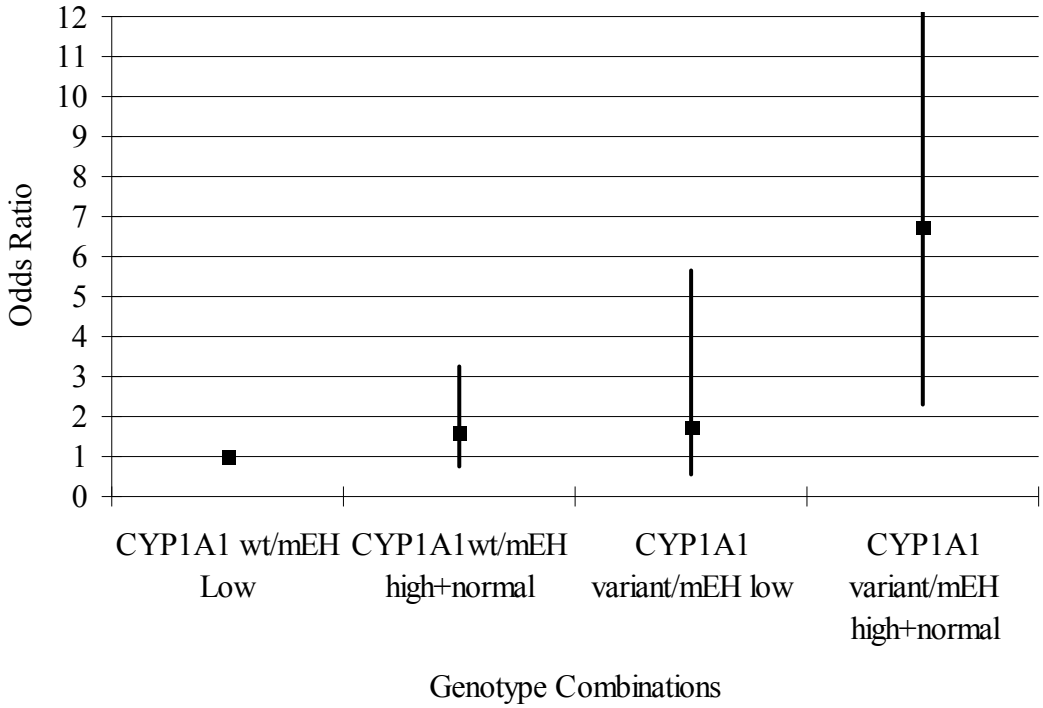

Figure 2. Squamous Cell Carcinoma Risk for Combinations of mEH and CYP1A1 Polymorphisms. Based on data from Lin *et al.* (2000)

Based on these findings, the higher activity mEH genotypes have greater ability to hydrolyze benzo(a)pyrene to the (-) benzo(a)pyrene 7,8-dihydrodiol than can be converted to the highly mutagenic BPDE by the higher activity CYP1A1. Despite these findings, Benhamou *et al.*, (1998) found that high mEH activity genotype's association with lung cancer was not modified by CYP1A1 or GSTM1 genotypes. Such findings could be explained by diet, small sample size, or the fact that

associations are more than the interaction between two biotransformation genes, depending on the exposure and population.

3.2.2 Chronic Obstructive Pulmonary Disease

Although the exact mechanisms behind the development of chronic obstructive pulmonary disease (COPD) are not fully understood, potential mechanisms of the disease are thought to include protease/antiprotease imbalance, inhibition of antiproteases by oxidants, such as tobacco smoke, and oxidant/free radical mediated cellular and tissue damage (Carp *et al.*, 1982; Church and Pryor, 1985; Garver *et al.*, 1986; Tetley, 1993; Farber, 1994; Smith and Harrison, 1997). Risk factors for COPD include both genetic factors (e.g., α_1-antitrypsin) and environmental factors (e.g., cigarette smoking) (Black and Kueppers, 1978; Bascom, 1991; Yim *et al.*, 2000). The primary environmental risk factor is smoking, but other risk factors include history of respiratory infection, air pollution, second-hand smoke, and occupational exposures to certain industrial pollutants (ALA, 2006). Given these risk factors and mechanisms, microsomal epoxide hydrolase may play a critical role in the development of these diseases. As available data shows, polymorphisms of mEH including the exon 3 variant alone and the combination of the combination of polymorphisms conferring low activity are susceptibility factors in COPD. However, associations between mEH genotype and risk of COPD are dependent on the level of exposures to tobacco, ethnicity, and gene-gene interactions.

3.2.2.1 Environmental Interactions

The mEH genotype has been shown to be associated with an increase or decrease in risk of COPD among Caucasians. Significant associations between COPD and mEH genotypes representing low activity have been observed in Caucasians from Spain, Russia, United Kingdom, and the U.S. (Smith and Harrison, 1997; Sandford *et al.*, 2001; Korytina *et al.*, 2003; Park *et al.*, 2005b; Rodriguez *et al.*, 2005). In smokers from Scotland, the very low activity genotype was associated with significant increases in risk for COPD and emphysema compared to all other predicted phenotypes with ORs of 4.1 (95% CI = 1.8-9.7) and 5.0 (95% CI = 2.3 – 10.9), respectively (Smith and Harrison, 1997).

In contrast to Caucasians, mEH may not be as great a susceptibility factor for COPD in Asian populations. In Japanese smokers, no significant associations were found between COPD or emphysema and mEH exon 3 and exon 4 polymorphisms (Takeyabu *et al.*, 2000; Yoshikawa *et al.*, 2000; Budhi *et al.*, 2003). Among Koreans who have a low prevalence rate of COPD and a high frequency of the slow mEH phenotype, no significant associations were observed between the mEH exon 3 and exon 4 genotypes and COPD (Yim *et al.* 2000). A lack of association between mEH and risk of COPD was also observed in smokers of Han nationality in North China (Cheng *et al.*, 2004).

Whereas mEH alone or in combination with a few other susceptibility factors (i.e., smoking) may not be significantly associated with the development of COPD in Asian populations, the genotype may play a role in the severity of the disease. While

the exon 3 homozygous variant was not associated with risk of COPD in Japanese COPD patients, Yoshikawa *et al.* (2000) did observe that the exon 3 variant was significantly higher in patients with severe COPD compared to mild cases (OR = 2.9; 95% CI = 1.1-7.4). Similar results were also observed in Taiwanese population with smoking-related COPD, where the exon-3 variant was significantly higher in patients with severe COPD compared to the homozygous wild-type genotype with an odds ratio of 7.5 (95% CI = 2.1-26.3) (Cheng *et al.*, 2004).

Although data on Asians supports that mEH is not a significant susceptibility factor for developing COPD, a couple of studies have found associations between the genotype and risk of COPD. Upon stratifying results according to smoking status (i.e., smokers, non-smokers and/or light smokers), significant associations between mEH and COPD risk were observed in Chinese non-smokers where the very slow activity genotype was associated with a significant increase in disease risk (OR = 1.89; 95% CI = 1.08-3.28) (Xiao *et al.*, 2004). No significant associations were observed in the smoking groups, however, a trend of decreasing risk was observed as pack years increased, suggesting the low-activity genotype is a protective factor among Chinese smokers. However, a recent study on the Han population from Southwest China, consisting of smokers, showed that the slow mEH activity genotypes was significantly higher in COPD patients compared to controls (Fu *et al.*, 2007), which is consistent with previous findings among Caucasian populations. The fast mEH genotypes were significantly lower in COPD patients than controls. Although these findings could be due to chance, such findings could be a result of

exposures to different environmental chemicals, dietary differences, and as will be discussed in the next section, gene-gene interactions.

3.2.2.2 Gene-Gene Interactions

Associations between mEH and COPD are also dependent on gene-gene interactions. This may in part explain the overall lack of association found in some Asian populations. When evaluating gene-gene interactions between mEH and GST genotypes, associations between mEH and COPD risk are observed. In Taiwanese smokers, Cheng *et al.* (2004) found that carriers of at least one variant exon 3 mEH allele compared to the high-activity genotypes was associated with a 2.3-fold (95% CI = 1.1-4.3) increase in COPD risk. When the low activity genotype was combined with GSTM1-null genotype, the relative risk of COPD was increased to nearly 4 fold ($p < 0.001$). When these genotypes were combined with the homozygous wild-type (ile105) GSTP1 genotype, the risk increased to 7 fold ($p < 0.001$) compared to individuals with no susceptible genotypes (Figure 3).

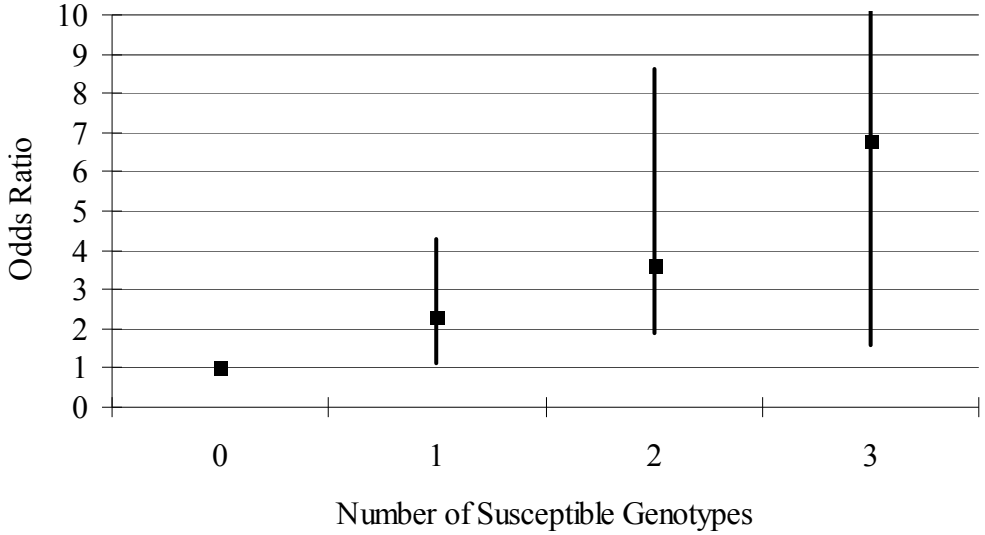

Figure 3. COPD risk for Combinations of Susceptible Genotypes. 0 = No susceptible Genotypes, 1 = at least 1 mEH exon 3 variant allele, 2 = mEH exon 3 variant + GSTM1-null, 3 = mEH exon 3 variant + GSTM1-null+GSTP1 Ile105/Ile105. Based on data from Cheng *et al.* (2004).

When evaluating the genotypes alone, only the GSTM1-null and mEH slow activity were associated with an increase in COPD risk. Researchers suggested that the combination these enzymes would lead to a weaker detoxifying capacity in the lungs against xenobiotics and ROS (Cheng *et al.*, 2004). Despite these observations, Yim *et al.* (2000) found no associations between the GSTM1, GSTT1 and mEH in COPD in Koreans. Such findings suggest that GSTP1 may be a determining factor in whether the combination of susceptible genotypes of mEH and GSTM1 are associated with increased risk of COPD and/or the risk of COPD is dependent on multiple susceptibility factors.

In addition to gene-gene interactions between mEH and GSTs, gene-gene interactions may occur between mEH and genes encoding antioxidants. Fu *et al.* (2007) found that the mEH slow genotype and heme oxygenase-1 variant (stress protein) were associated with a significant increase risk of COPD. Although the exact genetic mechanisms were uncertain, Sandford *et al.* (2001) found that individuals having the low-activity mEH genotype (homozygous His^{113}-His^{139}) and family history of COPD had a significantly higher risk of lung function decline (OR = 4.9; 95% CI 1.1-34.9, p = 0.04). Without consideration of family history, the risk for rapid lung function decline for low-activity genotypes (homozygous His^{113}-His^{139}) was 2.4 (95% CI = 1.1-5.4, p = 0.03).

3.5 Parkinson's Disease

Microsomal epoxide hydrolase has been shown to be expressed in tissue from the substantia nigra (Ahmadi *et al.*, 2000); therefore, it is possible that this enzyme may have a potential role in the development of PD. However, studies are too limited to make any firm conclusions regarding mEH's involvement in PD. One study on Swedish PD patients has shown that individuals homozygous for the low-activity isoform of mEH (113 H) were significantly associated with a 3.8-fold (95% CI = 1.2-11.9; p = 0.008) increase in risk of PD compared to individuals homozygous for the wild-type allele. These results were not observed in a larger study on non-Hispanic Caucasians in the U.S. (Farin *et al.*, 2001). This study found no overall association between either of the two mEH polymorphisms and risk of PD, and suggested the

reasons for the difference between these two studies may be due to sample size and/ or that any associations were obscured by the heterogeneous population.

3.6 Liver Disease

Studies have suggested that microsomal epoxide hydrolase (mEH) is involved in the detoxification of several xenobiotics known to cause disease and damage to the liver. These include, but may not be limited to aflatoxin B1, a potent liver carcinogen, and metabolites of ethanol metabolism (Guengerich and Davidson, 1982; Seidegard and DePierre, 1983; Guengerich *et al.*, 1998). Overall, the mEH genotype alone has shown little association with hepatic injury, but data suggests that in combination with other at-risk factors, the genotype may be associated with risk of hepatotoxicity.

3.6.1 Liver Cancer

The association between the mEH genotype and risk of hepatocellular carcinoma (HCC) is suggested to be dependent on other at-risk factors when evaluating associations between mEH genotypes and aflatoxin B1-related HCC. Whereas mEH may play a critical role in hydrolyzing the reactive intermediate of Aflatoxin B1 (AFB1) biotransformation, AFB1-8,9-exo-epoxide, studies have shown that the reaction is not rate-limiting (Johnson and Guengerich, 1997; Guengerich *et al.*, 1998). Therefore, the mEH genotype yielding the reduced activity enzyme (exon 3 mutant) may not influence the overall detoxification process. Additionally, GSTs can conjugate the reactive epoxides (Johnson and Guengerich, 1997; Guengerich *et al.*, 1998). Due to these factors, an association between the variant mEH genotypes

alone (i.e., exon 3 mutant and/or exon 4 wild-type alleles) and risk of aflatoxin-related HCC may be slight at best. This is supported in studies on biomarkers of aflatoxin exposure. Among African populations exposed to high levels of aflatoxin, several studies found no significant associations between aflatoxin-albumin adduct levels and the low-activity mEH allele (exon 3) (McGlynn *et al.*, 1995; Wild *et al.*, 2000; Dash *et al.*, 2007). However, McGlynn *et al.* (1995) reported a non-significant increase in adduct levels (albumin –AFB1) among carriers of the low activity allele, and Dash *et al.* (2007) found that exon 4 polymorphism conferring high mEH activity was associated with an increase in aflatoxin-albumin adduct levels. Whereas associations between adduct levels and mEH polymorphisms are inconclusive, they do suggest that the mEH genotype alone does not impact the overall aflatoxin-albumin adduct levels.

When evaluating the associations between mEH genotype and risk of HCC, the data shows the association between the genotype and risk of cancer is dependent on a combination of several at-risk factors. When considering the mEH genotype alone without consideration of other susceptibility factors, little or no association has been found between the variant and risk of HCC (McGlynn *et al.*, 1995; Wong *et al.*, 2000; Tiemersma *et al.*, 2001; McGlynn *et al.*, 2003; Kirk *et al.*, 2005). Despite these findings, significant exposure to aflatoxin may modify the association between mEH genotype and risk of HCC. This was suggested in a study by Tiersma *et al.* (2001) who found that the mEH slow activity genotype may increase the HCC risk in individuals exposed to high levels of aflatoxin, but not in low exposure individuals.

However, the interaction between aflatoxin exposure and genotype did not reach statistical significance.

Although data is limited, disease status shows to impact the association between mEH genotype and risk of HCC. This is supported in a small study by McGlynn *et al.* (1995) who found that carriers of the Hepatitis B surface antigen (HBsAG+) with at least one copy of the mEH variant exon 3 allele had a substantial increase in risk with an OR of 77.27 (95% CI = 8.9 - 665.8), compared to those carrying both wild-type alleles and no viral infection (HBsAg-) or the null alleles and no viral infection. As a comparison, the ORs for the mEH exon 3 wild-type/HBsAG+ group and mEH exon 3 variant/HBSAG- were 15.00 (95% CI = 1.2 - 184) and 3.3 (95% CI = 0.39 - 28.6). Confirming the impact of disease on the association between mEH genotype and risk of HCC, Sonzongni *et al.* (2002) found a statistically significant increase (OR 2.9; 95% CI = 1.0-4.6; p = 0.03) in Hepatitis C Virus (HCV)-related HCC among individuals carrying both mEH exon 3 variant alleles.

Whereas disease status and possibly high exposures to aflatoxins have shown to be a factor in determining whether the mEH genotype is associated with an increase risk of HCC, such findings were not observed by Kirk *et al.* (2005). In a study on Gambians with high exposures to aflatoxin, no significant association between mEH genotype and HCC risk were observed. When stratified according to HBV antigen (HBVAg), carriers of at least one exon 3 variant allele were associated with a nearly significant increase in HCC risk among HBsAg-negative carriers (OR =

2.49; 95% CI = 0.97 – 6.38). No increase in risk was observed in carriers of the HBvAg (OR = 0.96).

Although the previous results suggest that aflatoxin exposure and disease status may not play a significant role in modifying the association between mEH and HCC risk, this association may require additional susceptibility factors. When considering gene-gene interactions, mEH combined with the variant DNA-repair enzyme X-ray repair cross-complementing group 1 (XRCC1) and GSTM1 genotypes has been shown to be associated with a significant increase in HCC risk. Among Gambians exposed to high levels of aflatoxin, the variant mEH and XRCC1 genotypes were significantly associated with an increase in HCC risk (OR = 5.89; 95% CI = 1.36-25.6) (Kirk *et al.*, 2005). This increase in risk was higher than either genotype alone. When these susceptible genotypes were combined with the GSTM1 null genotype, the risk increased 14.7 fold (95% CI 1.27-169) (Figure 4).

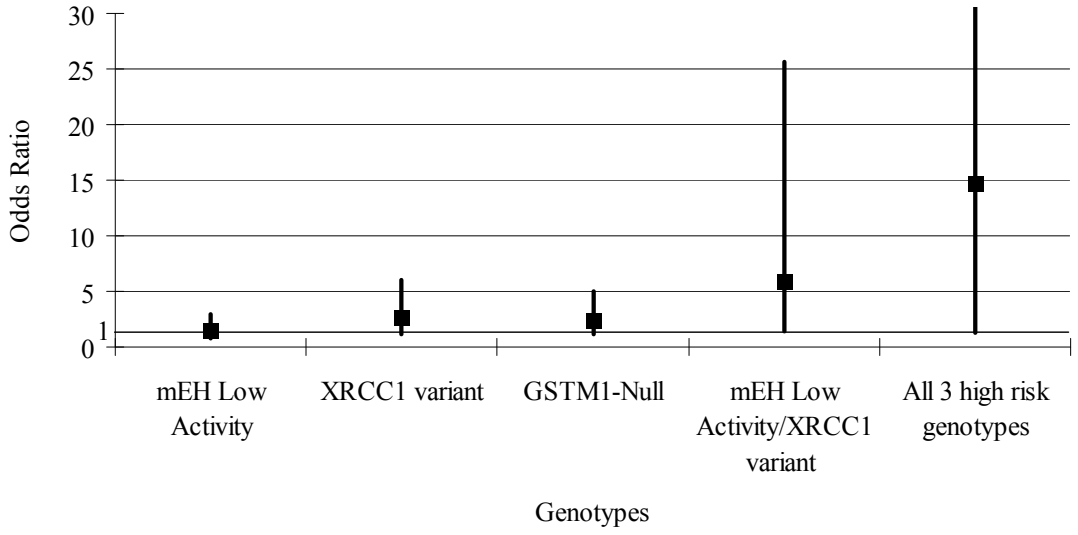

Figure 4. HCC risk for Combinations of mEH, GSTM1, and XRCC1 Polymorphisms. Based on data from Kirk *et al.* (2005)

3.6.2 Non-Cancer Diseases of the Liver

As for other diseases of the liver including chronic hepatitis, liver cirrhosis, and alcoholic liver disease (ALD), data is too limited to make any general conclusions. However, existing studies do support that disease status and high environmental exposures may impact the association between mEH genotype and risk of liver disease. The most comprehensive study by Sonzongni *et al.* (2002) found a significant association between the predicted very-low-activity mEH genotype and Hepatitis C Virus (HCV)-related cirrhosis. In individuals who had high alcohol consumptions (greater than 60 grams per day for more than 10 years), Wong *et al.* (2000) found no association between the mEH exon 3 variant genotype, but did find a significant association between the mEH exon 4 variant and risk of ALD. The authors suggested that the higher mEH activity may increase p450 activity resulting

in increased conversion of ethanol to acetaldehyde, which is more toxic. It was also suggested that the exon 4 variant may be in linkage disequilibrium with genetic risk factors for ALD (Wong *et al.*, 2000).

3.7 Summary

In general, without considering gene-gene and gene-environment interactions, associations between the mEH genotype are relatively inconsistent and do not support that mEH is a risk factor for disease. However, when these interactions are considered, significant associations are observed (Table 2). More importantly, these interactions explain the heterogeneity observed in the various studies on mEH polymorphisms and provide insight on the complexities associated with linking polymorphisms to disease.

Table 2. Summary of Associations Between mEH Genotypes and Disease

	Lung Cancer	COPD	PD	Liver Cancer	Liver Disease (Non-Cancer)
Low Activity mEH Genotypes	↑ Non-smokers and Short-term smokers ↓ Long-term Smokers	↑ Smokers and non-smokers	Uncertain	↑ Multiple susceptible Genotypes	Uncertain
High Activity mEH Genotypes	↑ Long-term Smokers	No data	Uncertain	No data	Uncertain

↑ = Increase in Risk, ↓ = Decrease in Risk

In the lung, the nature of the association between mEH genotype and risk of lung cancer is largely dependent on smoking exposure and duration. Among smokers, the variant genotypes conferring low enzymatic activity have been associated with significant decreases in lung cancer, and the high-activity genotype has been associated with an increase in cancer risk. However, the low- and high-activity mEH genotypes are possibly associated with increases and decreases in lung cancer risk, respectively, among non-smokers, where carriers of the low activity genotype are unable to hydrolyze reactive intermediates of environmental pollutants. Such associations, especially among smokers, may be further compounded by gene-gene interactions, whether it is due to decreased activity in a complementary enzyme involved in detoxification (i.e., GST) or an increase in activity of an enzyme that is involved in the bioactivation of intermediates of mEH hydrolysis.

In addition to smoking and gene-gene interactions, other factors may influence these associations. Associations between mEH and lung cancer may differ according to ethnicity where African-Americans and Caucasians having the predicted low-activity genotype may have significant decreases in lung cancer risk compared to intermediate- and high-activity genotypes. Cumulative exposures may alter these associations, where long-term exposure to tobacco smoke presents a more significant risk than susceptible genotypes. Furthermore, target tissue may affect this interaction, where the susceptible genotypes may only be a risk factor in certain tissues.

Although the low-activity mEH genotype may confer protection against the development of lung cancer in smokers, this same genotype may be a risk factor for

COPD. In this instance, the low-activity genotype is less able to detoxify products of oxidative stress resulting from tobacco smoke exposure. Despite this association, the association between the low mEH activity genotype may also be dependent on interactions with other genes encoding enzymes capable of detoxifying reactive products of oxidative stress and with antioxidants that may contribute to protection or susceptibility to disease. This is especially true in Asian populations where the risk of developing COPD is lower compared to other ethnicities (i.e., Caucasians).

As for the other diseases evaluated in this thesis, little or no data is available to accurately or fully evaluate any potential associations. However, studies on the liver do support that mEH is associated with liver disease in combination with multiple at-risk factors including exposures to high levels of liver carcinogens (i.e., aflatoxin), hepatitis, and other susceptible genotypes.

Chapter 4: NAD(P)H-Quinone Oxidoreductase 1

4.1 Introduction

This chapter will evaluate the impact of polymorphisms in the gene encoding NQO1 on risk of diseases of the lung, bladder, and liver as well as Parkinson's disease. As will be shown, associations between NQO1 are dependent on gene-gene and gene-environment interactions. This section will also show that the types of associations between NQO1 and risk of disease (i.e., risk factor, protective factor or neither) are specific to particular target tissues and disease.

4.2 Lung Cancer

Like microsomal epoxide hydrolase, NQO1 has dual functions of both activating and detoxifying carcinogens (Sunaga *et al.*, 2002). In the lung, it is likely to detoxify DNA-adduct forming quinones resulting from exposures to tobacco smoke (Joseph *et al.,* 1994). Also, NQO1 can activate nitroaromatic compounds and heterocyclic amines present in tobacco smoke (De Flora *et al.*, 1994; Saldivar *et al.*, 2005). Consequently, it is possible for the NQO1 polymorphism to have an association that can be linked to an increase or decrease in lung cancer risk. Such associations are dependent on other factors, including exposures to environmental toxins, gender, ethnicity, age, and target tissue. Also, such associations may depend on an interaction of these factors, as well as whether individuals are homozygous or heterozygous for the variant allele.

Smoking status has shown to be a determining factor in whether the NQO1 genotype is a risk factor or protective factor. In smokers, carriers of the variant allele

have shown decreases in lung cancer risk, whereas non-smokers carrying the variant allele have shown to be at a significant increase in cancer risk (Wiencke *et al.*, 1997; Bock *et al.*, 2005; Saldivar *et al.*, 2005). Despite these results, the variant genotype has also been associated with an increase in lung cancer in smokers (Lewis *et al.*, 2001; Xu *et al.*, 2001). However, such discrepancies may be a result of study design as smoking characteristics (i.e., the length of exposure and intensity) may be more important in determining whether the NQO1 genotype plays a role as a risk factor or protection factor than smoking status alone. Among smokers, the NQO1 variant genotype may be a risk factor for lung cancer in the short-term, but in the long-term, it a protective factor. While non-significant, Xu *et al.* (2001) found that as smoking years decreased in former smokers, cancer risk increased for homozygous NQO1 variants carriers. Smoking intensity (cigarettes per day) had no significant impact on risk estimates for homozygous variants, which is likely due to the fact that less than 3% of the population were homozygous for the variant genotype More importantly, in the heterozygous groups consisting of former smokers, a similar trend was observed; however, significant associations with lung cancer were observed that were dependent on smoking time and intensity (Xu *et al.*, 2001). Intense smoking was associated with a significant increase in risk for people who smoked 30 years or less and the risk increases as years of smoking decrease, suggesting NQO1 decreased ability to detoxify high levels of carcinogens (PAH-quinones). An OR of 3.91 (95% CI = 1.52-10.0) was estimated for former smokers who smoked 40 cigarettes/day for only 1 year. The risk decreased to 1.65-fold (95% CI = 1.05-2.58) for individuals of

the same smoking intensity who smoked 30 years (Xu *et al.*, 2001). After 30 years, no significant associations were observed (Figure 5).

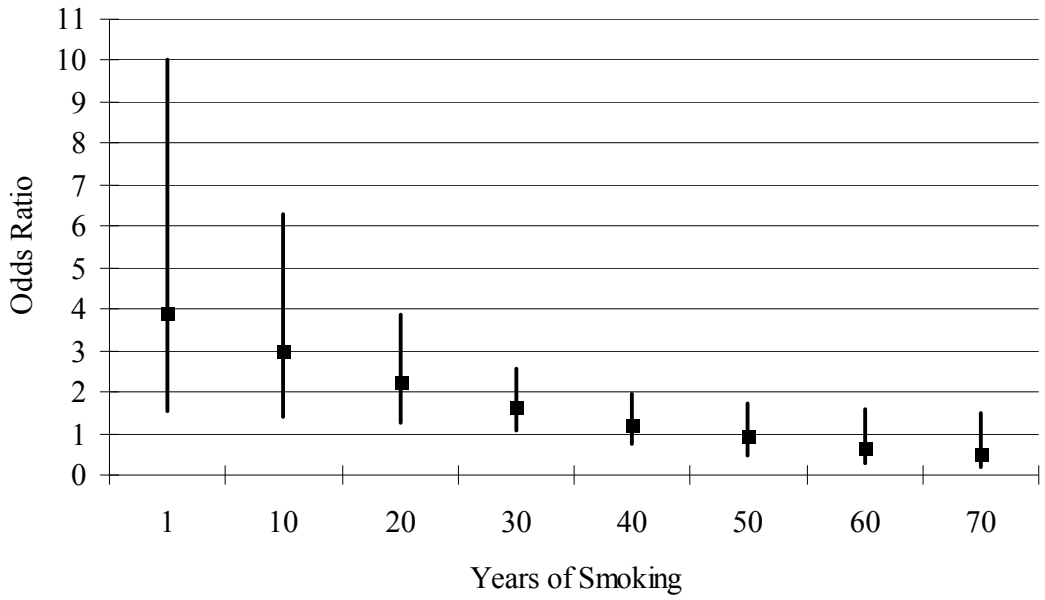

Figure 5. Lung Cancer Risk for Former Smokers (40 cigarettes/day) with the Heterozygous C/T compared to C/C NQO1 genotype. Based on data from Xu *et al.* (2001)

In moderate (20 cigarettes/day) and light (5 cigarettes/day) intensity smokers having the C/T genotype, a trend of decreasing risk was observed. In long-term light and moderate smoking groups, the heterozygous variants had a significant decrease in risk compared to the homozygous wild-type group with ORs of 0.26 (95% CI = 0.12-0.58) and 0.61 (95% CI = 0.40-0.94), respectively after 40 years of smoking (Figures 6 and 7). No significant associations were observed in the long-term heavy smoker group.

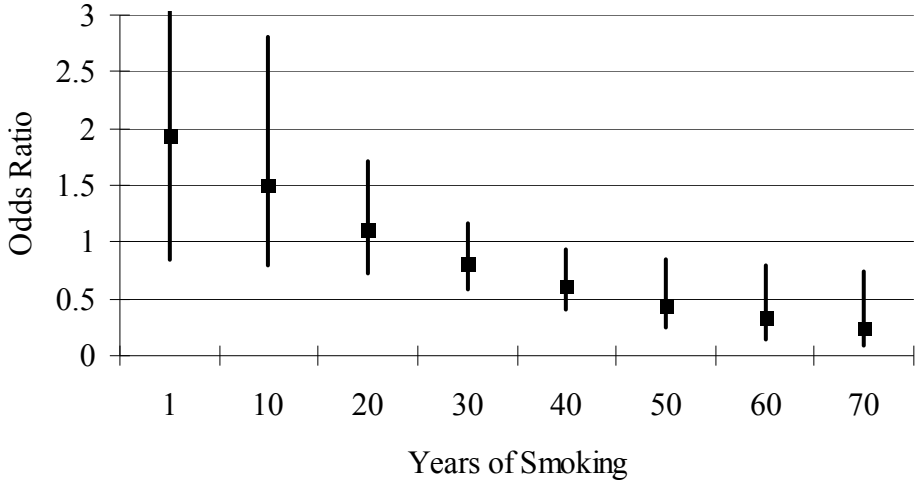

Figure 6. Lung Cancer Risk for Former Smokers (20 cigarettes/day) with the NQO1 C/T genotype compared with the homozygous wild-type (C/C) genotype. Based on data from Xu *et al.* (2001)

Figure 7. Lung Cancer Risk for Former Smokers (5 cigarettes/day) with the NQO1 C/T genotype compared with the homozygous wild-type (C/C) genotype. Based on data from Xu *et al.* (2001)

In addition to smoking duration and intensity, ethnicity may too play a role in associations between the NQO1 genotype and lung cancer risk. Overall, Asian populations tend to show stronger associations between NQO1 genotype and lung cancer risk compared to Caucasians and other ethnic groups. In combined groups of non-smokers and smokers, Japanese populations have shown significant associations between the variant genotype and decrease in cancer risk whereas Caucasians, African-Americans, and Hispanics show no significant associations (Chen *et al.*, 1999; Hamajima *et al.*, 2002; Sunaga *et al.*, 2002; Alexandrie *et al.*, 2004; Bock *et al.*, 2005; Saldivar *et al.*, 2005). Associations in combined groups were also not observed in Chinese lung cancer patients (Yin *et al.*, 2001).

When evaluating ethnicity after stratifying data according to smoking status, differences are also observed between the various ethnicities. In African-American smokers, no associations between genotypes and lung cancer risk have been observed (Wiencke *et al.*, 1997; Bock *et al.*, 2005; Saldivar *et al.*, 2005). Similar results were also observed in Caucasian smokers from Finland, Denmark, Sweden, and U.S. (Chen *et al.*, 1999; Alexandrie *et al.*, 2004; Bock *et al.*, 2005; Lawson *et al.*, 2005; Saldivar *et al.*, 2005; Sorensen *et al.*, 2005). In a population of lung cancer patients from Hawaii, only Japanese individuals homozygous for the variant allele were associated with a significant decrease in cancer risk (Chen *et al.*, 1999). Hawaiians and Caucasians with the same genotype had a non-significant decrease in cancer risk. While ethnicity may play a role, the small percentage of homozygous variants in Hawaiians and Caucasians may contribute to this discrepancy (Chen *et al.*, 1999).

Contrary to these findings, a group of non-smoking Caucasians in the U.S. carrying at least one variant genotype had a significant increase in lung cancer risk (OR =1.80; 95% CI = 1.03–3.13) (Saldivar *et al.*, 2005), whereas Xu *et al.* (2001) found significant associations between smoking and lung cancer risk NQO1 genotype in Caucasian former smokers. Based on these observations, NQO1's role in lung cancer is likely dependent on numerous susceptibility factors.

Cancer histological type may too be a modifying factor for associations between lung cancer and NQO1 genotype and may be a reason for conflicting findings and lack of associations. In Japanese smokers and non-smokers, the NQO1 homozygous wild-type high-activity genotype has been associated with a significant increase in adenocarcinoma with an OR of 2.15 (95% CI = 1.03-4.48) when compared to the homozygous variant genotype (NQO1-Ser/Ser) (Sunaga *et al.*, 2002). These findings are consistent with NQO1's ability to bioactivate heterocyclic amines. Similar results were also observed in Taiwanese smokers with an OR of 2.93 (95% CI = 1.23-7.02) (Lin *et al.*, 1999). Despite these findings, no associations were found between genotype and the broader category of non-small cell carcinoma among Chinese (Chan *et al.*, 2005). Among Caucasians, generally no associations have been found between NQO1 genotypes and histology type (Alexandrie *et al.*, 2004; Lawson *et al.*, 2005; Saldivar *et al.*, 2005). However, in the United Kingdom individuals carrying at least one variant NQO1 allele had a significant increase in small-cell cancer with an OR of 3.8 (95% CI = 1.19-12.1) (Lewis *et al.*, 2001). Among heavy smokers, the risk increased 12.5-fold (95% CI = 2.1-75.5). This same study found no

association between NQO1 genotypes and non-small cell cancer risk. Whereas these results might suggest that the NQO1 genotypes may not be associated with non-small cell cancer in Caucasians, non-significant increases in squamous cell carcinoma were observed in other studies on Caucasian populations (Xu *et al.*, 2001; Alexandrie *et al.*, 2004).

Gender may also impact the association between NQO1 genotype and lung cancer risk. Sex-related differences in bioactivation of specific compounds by other metabolic enzymes (e.g., P450) may impact such associations (Saldivar *et al.*, 2005). In a population of Caucasian, Hispanic, and African-American women carrying at least one variant NQO1 allele , the risk of lung cancer increased significantly compared to women with homozygous wild-type genotype (OR = 1.89; 95% CI = 1.35-2.65) (Saldivar *et al.*, 2005). No associations between genotype and cancer were found in men from the same study. However, no associations between NQO1 genotype and lung cancer risk were observed in another population of Caucasian and African-American women (Bock *et al.*, 2005).

In addition to environmental exposures, ethnicity, and histology, NQO1's association with cancer risk may be age-dependent. In Caucasians and African-Americans smokers and non-smokers carrying at least one copy of the variant allele who were diagnosed after 50 years of age, the risk of lung cancer decreased significantly (OR = 0.48; 95% CI = 0.27-0.87) (Bock *et al.*, 2005). Note, when stratified according to ethnicity, only the Caucasian population was associated with a significant decrease in cancer risk. Also, no significant associations were found in

individuals diagnosed before the age of 50 even after stratifying according to race, gender, and smoking status. Bock *et al.* (2005) suggested that protective effect is related to the decreased activation of carcinogens, including components of environmental tobacco smoke. Furthermore, the younger group may not have had significant enough exposures in combination with the NQO1 genotype to influence risk levels, therefore, the genetic mechanism behind early onset cancer may be independent of NQO1 (Bock *et al.*, 2005).

Though data is limited, gene-gene interactions likely increase susceptibility of lung cancer among carriers of the variant NQO1 genotype. Alexandrie *et al.* (2004) found a non-significant increase in squamous cell carcinoma for CYP1A1 variants and NQO1 variants. In another study, the NQO1 wild-type and GSTT1-null genotypes were associated with an increased risk of adenocarcinoma, which was significant in smokers, but not in non-smokers (Sunaga *et al.*, 2002). The authors suggested that the NQO1 and GSTT1 metabolic pathways overlap, and further concluded that tobacco carcinogens activated by NQO1 are not being detoxified by GSTT1. Of interest is that this study did not find any association between NQO1 and variants of other genotypes including CYP1A1 and GSTM1. However, this may have been likely due to the histology type evaluated in the study, where it is believed that heterocyclic amines are the primary cause of smoking related adenocarcinoma. Whereas, CYP1A1 and GSTM1 are primarly involved in biotransformation of PAHs that may more likely involved in squamous cell carcinoma.

4.3 Bladder

Although far fewer studies are available, a recent meta-analysis by Chao *et al.* ((Chao *et al.*, 2006) found no significant associations between the NQO1 genotype and risk of bladder cancer. However, data does suggest that NQO1 genotype and its association with bladder cancer may be impacted by environmental interactions and gender. In addition, the studies suggest that the type of association between the genotypes and bladder cancer risk, whether they are protective or susceptibility factors, depend on significant exposure to bladder carcinogens (e.g., smoking).

Several studies on Caucasians have suggested that the NQO1 genotype alone may be associated with bladder cancer risk. In a study by Schulz *et al.* (Schulz *et al.*, 1997), researchers found that the NQO1 variant allele was associated with a 3.6-fold increase in urothelial carcinoma risk among German patients. Similar results (i.e., increase in risk) were also observed by Hung *et al.* (2004a) in non-smokers in a highly industrialized area of Northern Italy and by Park *et al.* (2003) in the U.S. However, Choi *et al.* (2003) found that the variant allele was associated with a protective effect, which supports that the wild-type NQO1's role as exerting harmful effects by producing active metabolites, reactive oxygen species, or activating heterocyclic amines present in cigarette smoke (Choi *et al.*, 2003). Of course, this role is dependent on the substrate (Park *et al.*, 2003; Terry *et al.*, 2005). Additionally, no associations, regardless of smoking status, have been observed in other studies (Choi *et al.*, 2003; Hung *et al.*, 2004a; Sanyal *et al.*, 2004; Terry *et al.*, 2005; Chao *et al.*, 2006).

Given such findings, gene-environment and gene-gene interactions likely play a critical role in associations between NQO1 genotype and bladder cancer. In fact, smoking explains some of the differences observed between the studies, suggesting an association between the NQO1 polymorphism and bladder cancer risk. Also, results support the notion that the NQO1 variant and wild-type enzymes' role as a protective factor or susceptibility factor depends on the length of exposure.

Overall, the variant NQO1 has shown to be a protective factor among smokers and a potential risk factor among non-smokers. When stratifying results according to smoking status, Moore *et al.* (Moore *et al.*, 2004) found that the variant genotypes (CTs and TTs) were associated with a decrease in bladder cancer risk among smokers (OR = 0.55; 95% CI = 0.22–1.09; $p = 0.09$) and that the variant among non-smokers may be associated with an increase in cancer risk (OR = 3.32; 95% CI = 1.18-9.39). More importantly results from this study indicated a gene-environment interaction between the NQO1 wild-type and smoking. Smokers homozygous for the NQO1 wild-type alleles had a significant increase in risk compared to non-smokers of the same genotype (OR = 8.58; 95% CI = 2.73–27.0; $p < 0.001$). In comparison, a significant increase in risk of bladder cancer was not observed in smokers carrying at least one variant NQO1 compared to non-smokers of the same genotype. Similar results were also observed by Choi *et al.* (2003).

In contrast to the studies by Moore *et al.* (2004) and Choi *et al.* (2003), Park *et al.* (2003) found an increase in cancer risk among smokers carrying the variant alleles (CT or TT). However, a significant increase in risk was only observed in individuals

smoking less than 20 years (OR = 2.59; 95% CI = 1.02-6.57). Although Park *et al.* (2003) suggested that environmental effects may have masked the genetic effects in heavy smokers (i.e., > 20 years smoking), it is possible that the mechanism by which NQO1 is a susceptibility factor differs according to the length of smoking, similar to that found in the lung. In the short-term smokers the variant is a risk factor, but over the long-term with repeated exposure it is a protective factor.

In addition to smoking, gender may also play a role in the development of bladder cancer and be modified by the NQO1 polymorphism. Park *et al.* (2003) found a statistically significant increase in bladder cancer among men (OR = 1.75; 95% CI = 1.08-2.85), but not women whom carried the variant alleles (CT or TT), regardless of smoking status.

Despite these associations, several studies have not found associations between the NQO1 and bladder cancer risk among smokers (Hung *et al.*, 2004a; Sanyal *et al.*, 2004; Terry *et al.*, 2005). Additionally, the increases in bladder cancer risk have been observed in non-smokers, but not in smokers from the same population (Hung *et al.*, 2004a). Although these observations may serve to reject data finding associations between NQO1 and bladder cancer risk, such observations support that these links are more complex than a combination of a couple susceptibility factors.

4.4 Parkinson's Disease

Precursors of oxidative stress that may lead to PD may include endogenous and exogenous factors such as *o*-quinones of catecholamines and neurotoxic pesticides (Tanner, 1989; Baez *et al.*, 1997; Ascherio *et al.*, 2006; Brown *et al.*,

2006). If NQO1 is involved in the detoxification of catecholamine-derived quinones, reduced or lack of activity in this enzyme could potentially contribute to the development of PD. However, like mEH, there are too few studies to make any firm conclusions regarding any potential association, but data does show that gene-environment and gene-gene interactions may affect the association between NQO1 genotype and PD. Overall, studies do not show consistent associations between the NQO1 genotype and risk of PD. Two studies conducted in China have found that the allelic variants were significantly higher in patients with PD (Shao *et al.*, 2001; Jiang *et al.*, 2004). In the Jiang *et al.* (2004) study, researchers found that the homozygous variant genotype was associated with a 2.2-fold increased risk of PD ($p = 0.004$). When stratified according to age at onset, the variant genotype was only associated with an increased risk in PD among late-onset PD with an OR of 2.67 ($p = 0.001$). Shao *et al.* (2001) also found that the variant allele to be significantly higher in PD patients with an OR of 3.8 ($p < 0.05$). Despite these observations, three other studies have found no overall association between the NQO1 genotype and PD (Harada *et al.*, 2001; Okada *et al.*, 2005; Fong *et al.*, 2007).

Such heterogeneity between these studies is perhaps a result of gene-environment and gene-gene interactions. Exposure to pesticides and other neurotoxic chemicals are risk factors for PD and in combination with the variant NQO1 allele could contribute to increase risk of PD. This is supported in the study by Fong *et al.* (2007) who found no overall association between the NQO1 variant and increase risk of PD. When cases were stratified according to exposure to pesticides, the NQO1

variant genotype was associated with increase risk of PD (OR = 2.49; 95% CI = 1.18-5.26).

In addition to pesticide exposure, gene-gene interactions could impact the association between the variant NQO1 genotype and PD risk. The combination of the variant NQO1 and manganese-containing superoxide dismutase (MnSOD) genotypes was associated with a significant increase in individuals exposed to pesticides (OR = 4.09; 95% CI = 1.16-10.64) (Fong *et al.*, 2007). In addition to interactions with MnSOD, the combination of the variant NQO1 genotype and high-activity MAO-B yielded an increase in PD risk with an OR of 5.7 (Shao *et al.*, 2001).

4.5 Liver

At this time, there is a lack of data on the impact of NQO1 on polymorphisms in liver disease among humans. However, NQO1 may play a critical role during periods of hepatic oxidative stress and damage (Chan *et al.*, 2001; Aleksunes *et al.*, 2006). Elevations in NQO1 mRNA in rodents have been observed following exposures to hepatotoxicants, such as acetaminophen, carbon tetrachloride, and bromobenzene (Heijne *et al.*, 2004; Aleksunes *et al.*, 2006). Therefore, a loss of activity of this enzyme could result in greater susceptibility to liver disease.

4.6 Summary

Overall, the NQO1 variant genotype may be associated with a slight increase in risk of diseases of the lung and bladder. However, the nature of this association is dependent on environmental factors and target tissue. In regard to non-smokers, the variant allele is associated with an increase in risk of developing bladder and lung

cancer (Table 3). In smokers, the variant NQO1 genotype has been associated as both a risk and protective factor, which is dependent on the duration of smoking (Table 3). In short-term smokers, the NQO1 variant genotype shows to be a risk factor similar to that of non-smokers, but in long-term smokers it is a protective factor.

Table 3. Summary of Associations Between NQO1 Genotype and Disease

	Lung Cancer	PD	Bladder Cancer	Liver Disease
NQO1 Variant	↑ Non-smokers and Short-term smokers ↓ Long-term Smokers	↑ Risk Pesticide Exposure and/or combination with other susceptible genotypes	↑ Non-smokers and Short-term smokers ↓ Long-term Smokers	Uncertain
NQO1Wild-Type	↑ Long-term Smokers	No Data	↑ Long-term Smokers	Uncertain

↑ = Increase in Risk, ↓ = Decrease in Risk

Though data is limited the NQO1 variant likely plays a role in the development of PD. Whereas the disease is dependent on numerous factors, NQO1 may play a role in detoxifying *o*-quinones of catecholamines and pesticides that could contribute to the development of PD. This was supported in one study that found a significant association between NQO1 and PD only after stratifying the study group according to pesticide exposure (Fong *et al.*, 2007). Additionally, gene-gene interactions between the NQO1 variant and other susceptible genotypes increase the risk of developing PD.

In regards to the liver, it is uncertain whether the NQO1 variant is a susceptible genotype due to a lack of data. However, the variant may be a risk factor in diseases of the liver especially in combination with other at risk genotypes given the liver is a rich source of enzymes.

Chapter 5: Glutathione *S*-Transferase

5.1 Introduction

This chapter will evaluate the impact of polymorphisms in the genes encoding GSTM1, GSTT1 and GSTP1 on risk of diseases of the lung, liver, and bladder, as well as Parkinson's disease. A considerable amount of research has gone into investigating the associations between GSTs and diseases of these organs and PD. Given the amount of research conducted, the associations between GST genotypes and risk of lung disease provide good examples on the overall association between individual polymorphisms on disease risk. More importantly this chapter will show how these associations are dependent on and/or modified by gene-gene and gene-environment interactions.

5.2 Lung

The role GST polymorphisms play in the development of lung disease has been well studied, especially in regards to lung cancer. Contrary to their relative expression levels where GSTP1 is strongly expressed in the respiratory epithelium and GSTM1 is low (Cantlay *et al.*, 1994), GSTM1 may play a greater role in the development of lung disease than GSTP1. Note, this is limited to our current understanding of the polymorphisms within the genes encoding these enzymes. Polymorphisms could exist within the GSTP1 gene that may be very rare and not detected in the typical study group that could have significant impact on the activity of the enzyme. Anyhow, current data shows that GSTM1 may play a more

significant role in disease of the lung compared to the other two GSTs evaluated in this thesis (i.e., GSTT1 and GSTP1).

5.2.1 Lung Cancer

5.2.1.1 GSTM1

Overall, data supports that the GSTM1-null genotype is associated with a slight increase in risk of lung cancer (Houlston, 1999; Benhamou *et al.*, 2002; Ye *et al.*, 2006). In the most recent meta-analysis combining the results from 119 studies, Ye *et al.* (2006) found that the GSTM1-null genotype was associated with a slight increase in lung cancer risk with an OR of 1.18 (95% CI = 1.14 -1.23). Although publication bias may have contributed to these findings, considering large studies have found no significant associations, differences have been observed between the studies. As will be discussed in this section, the association between GSTM1 genotype and risk of lung cancer is dependent on a combination of factors. Additionally, the GSTM1-null genotype may exert its effect on lung cancer indirectly via blood borne metabolites from the liver where GSTM1 is highly expressed (Houlston, 1999).

When considering smoking status and its impact on the association between GSTM1 genotype and lung cancer risk, differences are generally not observed between smokers and non-smokers. In fact, it has been suggested that the GSTM1 polymorphism effect on lung cancer risk in nonsmokers is similar to that of smokers (Malats *et al.*, 2000). In a pooled analysis of 21 studies, Benhamou *et al.* (2002) did not find any interaction between smoking status and the GSTM1-null genotype. Even

after stratifying non-smokers and smokers according to histology, ethnicity, and gender, no significant differences were observed.

Although smoking status may not impact the association between lung cancer and GSTM1-null genotype, without other interactions (i.e., gene-gene interactions), the GSTM1-null genotype may be associated with lung cancer risk when considering exposures to other lung carcinogens. In nonsmokers of mixed European and South American descent, the GSTM1 genotype modified the association between exposures to occupational carcinogens and other environmental exposures and risk of lung cancer (Malats *et al.*, 2000). Though the study groups were small and results were not significant, individuals exposed to indoor wood combustion greater than 20 years, or occupational carcinogens carrying the null genotype had greater increases in lung cancer risk compared to individuals with GSTM1-present genotype.

Associations between the GSTM1-null genotype and lung cancer risk have also been observed among individuals exposed to radon, which induces oxidative stress via ionizing radiation (α-particles) (Narayanan *et al.*, 1997; Alavanja, 2002). A study by Bonner *et al.* ((Bonner *et al.*, 2006) found that the GSTM1-null genotype was associated with a 3.41-fold increase (95% CI = 1.10 – 10.61) in lung cancer compared to the GSTM1-present genotype among patients exposed to high levels of radon gas (> 121 Becquerel per cubic meter). However, the results showed that the association was dose-dependent, given that no significant increase in lung cancer risk was observed in GSTM1-null individuals compared to GSTM1-present individuals at lower levels of radon (< 121 Becquerel per cubic meter) (Figure 8).

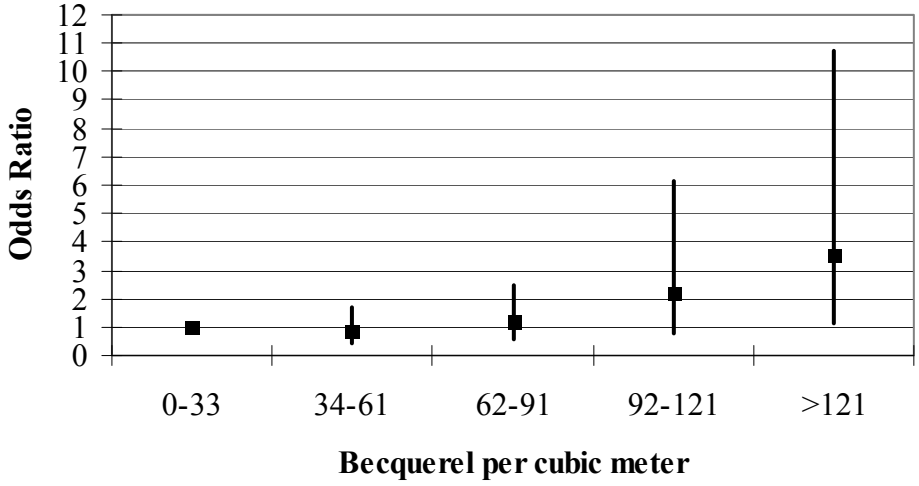

Figure 8. Relative Risk for Lung Cancer for GSTM1-null versus GSTM1-present genotypes for varying levels of Radon exposure. Based on data from Bonner *et al.* (2006).

Ethnicity may play a minor role in GSTM1-null's association with lung cancer risk. In the two major ethnicity groups studied in the meta-analysis by Ye *et al.* (2006), which included individuals of European continental ancestry and East Asian, the null genotype's association with lung cancer differed between each group. Among the European continental ancestry group, the GSTM1-null genotype has shown no association with an increase in lung cancer risk. The relative risk for East Asians with a null genotype frequency of 30% was slightly greater than the overall OR for the entire study, which was 1.18. Although the number of studies was much less, the OR for the group consisting of individuals not falling in the previous categories (i.e., African-Americans, Mexican-Americans, etc.), the relative risk for

individuals in the null genotype was approximately equal to the study-wide OR (Ye *et al.*, 2006).

In regards to other ethnicities or populations from specific regions, where the number of studies are low, data shows some variability. In lung cancer patients from Turkey, the GSTM1-null genotype was significantly associated with lung cancer with an OR of 4.14 (95% CI = 2.36-7.27) (Pinarbasi *et al.*, 2003). Consistent with other findings on smoking, no significant association was found between smoking and the null genotype. Ethnic differences are also observed in a population of Chileans, where the relative risk for lung cancer associated with the GSTM1-null genotype was 2.46 ($p = 0.004$) (Quinones *et al.*, 2001).

Despite the overall weak association that GSTM1 genotype may have with the risk of lung cancer, the genotypes association with lung cancer risk may be significantly influenced by diet. Isothiocyanates derived from cruciferous vegetables (e.g., broccoli) are thought to inhibit carcinogenesis by either protection against oxidative damage or inhibition of apoptosis (Yu *et al.*, 1998). Experimental evidence has shown that isothiocyanates induce the expression of phase-I and phase-II enzymes via the antioxidant/electrophile response element, but inhibit P450s (Zhang and Talalay, 1998; Nho and Jeffery, 2001; Lampe and Peterson, 2002). It is thought that glutathione conjugation is responsible for elimination of anticarcinogenic substances, such as isothiocyanates (Kolm *et al.*, 1995).

Available data does indicate that GSTM1-null genotype does modify the protective factor of isothiocyanates in the lung. London *et al.* (2000b) found that lung

cancer risk decreased significantly in Chinese men (smokers and non-smokers) having the null genotype with detectable levels of isothiocyanates compared to individuals with undetectable levels of isothiocyanates (OR = 0.38; 95% CI = 0.23-0.62), but no difference in lung cancer risk was observed between the GSTM1-present genotypes with detectable and undetectable isothiocyanate levels (Figures 9 and 10).

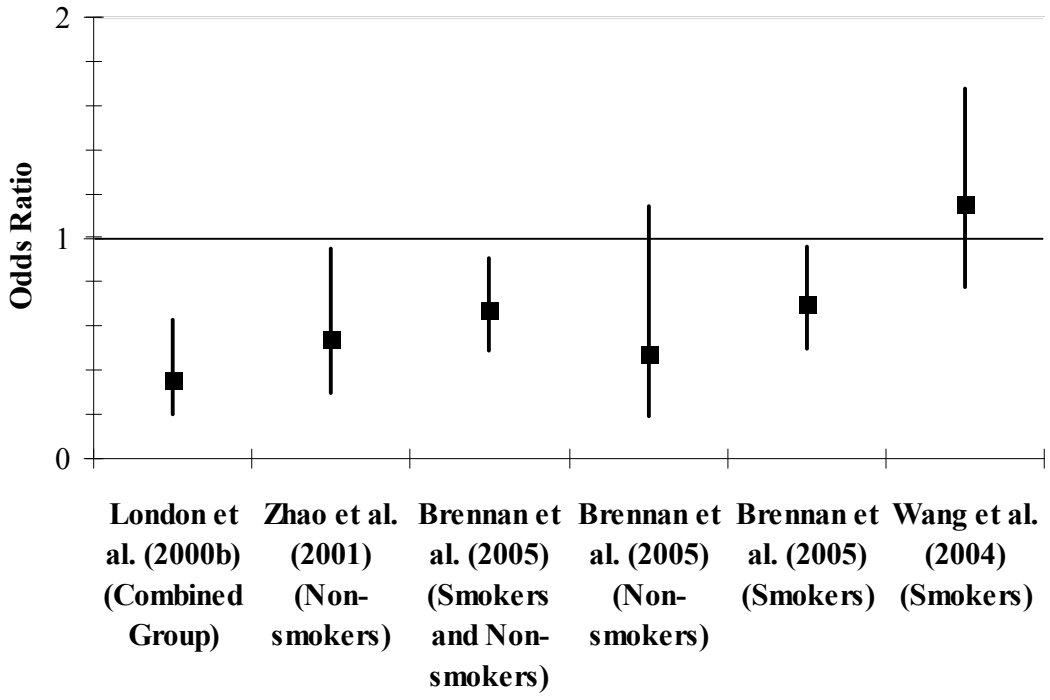

Figure 9. Relative Risk of Lung Cancer for High versus Low Dietary Intake of Isothiocyanates among GSTM1-null Individuals

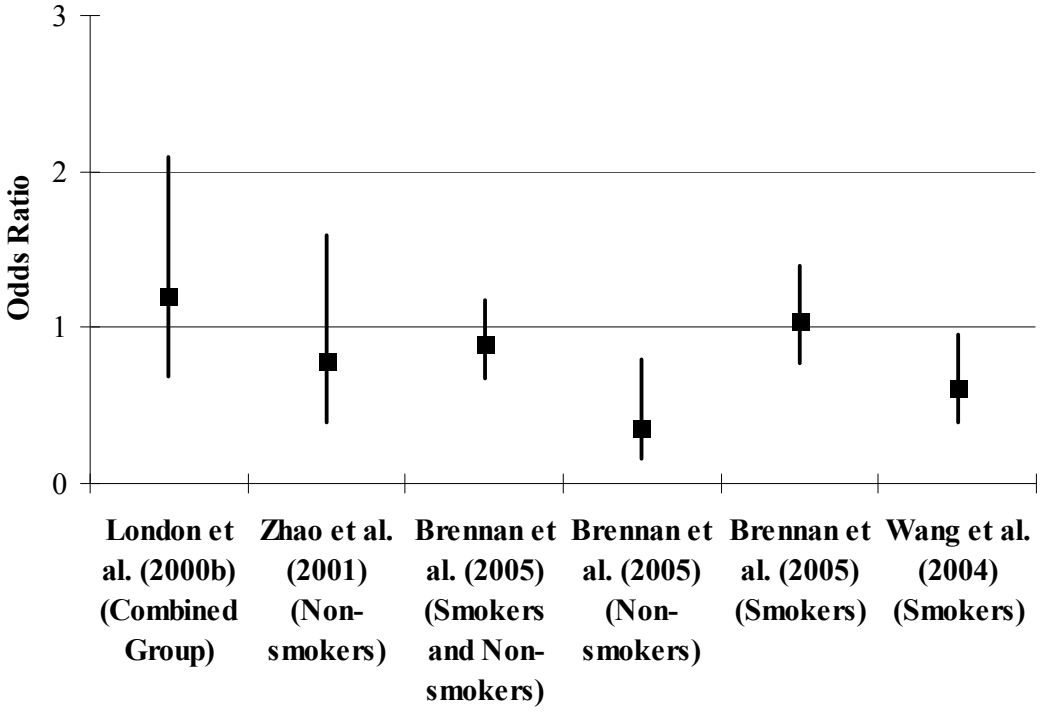

Figure 10. Relative Risk of Lung Cancer for High versus Low Dietary Intake of Isothiocyanates among GSTM1-present Individuals

Interestingly, GSTM1-null individuals with undetectable levels of isothiocyanate in urine had an increased risk of lung cancer compared to wild-type individuals when adjusted for smoking (OR = 2.35; 95% CI = 1.02-5.41). However, in individuals with detectable levels of isothiocyanates, the GSTM1-null genotype was associated with a decreased risk of lung cancer compared to the non-null group (OR = 0.60; 95% CI = 0.43- 0.84) (London *et al.*, 2000b) (See Figure 11).

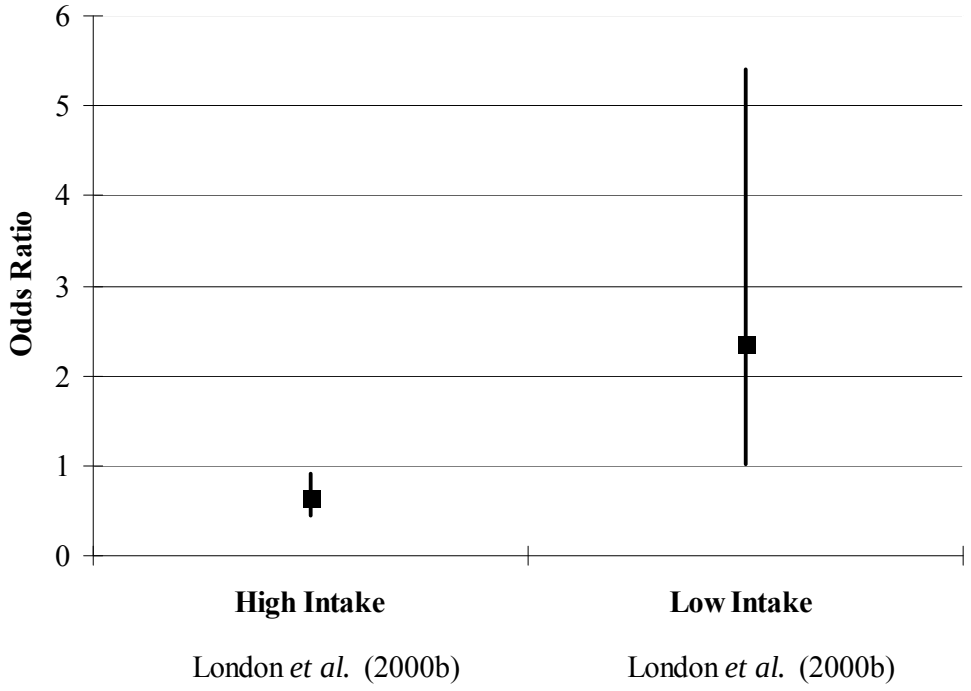

Figure 11. Relative Risk of Lung Cancer for GSTM1-null versus GSTM1-present genotypes in High and Low Isothiocyanate Intake

In a much larger study on central and eastern Europeans (*n* = 2141), a significant protective effect of the GSTM1-null genotype was also found in the high-cruciferous-vegetable-intake group compared to the low-and-medium-intake groups (OR = 0.67; 95% CI = 0.49-0.91) (Brennan *et al.*, 2005) (See Figure 9). High intake of cruciferous vegetables in the GSTM1 non-null group had no significant impact on risk in the group overall, but did have a significant decrease in risk for non-smokers (See Figure 10)

In non-smokers, dietary intake of isothiocyanates has also shown to impact GSTM1 association with lung cancer risk. In non-smoking Chinese women in

Singapore, high intake of isothiocyanates by GSTM1-null individuals reduced lung cancer risk by 50% compared to null individuals with low isothiocyanate intake (OR = 0.54; 95% CI = 0.30-0.95) (Zhao *et al.*, 2001). Risks were not significantly attenuated in non-null individuals with high intake of dietary isothiocyanates. Similar results were also observed by Brennan *et al.* (2005), however non-smokers having the GSTM1 gene with high dietary isothiocyanates had a significantly lower risk compared to the low intake group (See Figures 9 and 10).

Whereas the previous observations would suggest that diet modifies the association between the GSTM1-null genotype and lung cancer risk, dietary patterns among different populations should be considered. Wang *et al.* (2004) noted that the daily dietary intake of cruciferous vegetables by Chinese in the previous studies (London *et al.*, 2000b; Zhao *et al.*, 2001) was three times greater than the average U.S. intake. Among a group of mostly former and current Caucasian smokers from the U.S., high-cruciferous-vegetable intake only reduced lung cancer risk in individuals with GSTM1 present genotype among a population of Caucasians, which is consistent with the findings by Brennan *et al.* (2005). However, a decrease in risk for the GSTM1-null genotype with high cruciferous vegetable intake was not observed. The authors attributed such findings as possibly a result of dietary intake of cruciferous vegetables not being high enough to offset the elevated exposure to tobacco carcinogens in GSTM1-null individuals (Wang *et al.*, 2004). Despite these observations, another study on American Caucasian smokers by Spitz *et al.* (Spitz *et al.*, 2000) found that both the non-null and null genotypes with low dietary intake of

isothiocyanates had significant increases in cancer risk of lung cancer compared to the high intake GSTM1 non-null group. In support of previous findings by Brennan *et al.* (2005) and London *et al.* (2000b), no significant increase in cancer risk was observed in the high intake GSTM1-null current smokers group compared to the high intake GSTM1 non-null group, however, risks were not decreased as found in the London *et al.* (2000b) study. Perhaps this is due to dietary intake of isothiocyanates being greater in the Chinese population. Overall, these findings are consistent with the overall weak association between GSTM1 genotype and risk of lung cancer in Caucasian populations and suggest that that diet rather than GSTM1 genotype is a determining factor for lung cancer risk in Caucasians in the U.S and populations with low isothiocyanate intake. In populations with high isothiocyanate intake, the GSTM1-null genotype may confer a decreased risk of lung cancer compared to the non-null genotype.

In addition to environmental exposure and diet, gender may too affect the association between genotype and risk of lung cancer. Although many studies have found no difference in lung cancer susceptibility between men and women (Melikian *et al.*, 2007), a few studies shown that women at a given level of tobacco smoke exposure are at a higher risk of lung cancer compared to men (Risch *et al.*, 1993; Zang and Wynder, 1996; Gasperino and Rom, 2004). Such difference could be a result of greater P450 activity or hormonal effects (Zang and Wynder, 1996). In support of this, two studies found that women carrying the GSTM1-null genotype had a greater increase in risk of lung cancer (Alexandrie *et al.*, 1994; Tang *et al.*, 1998).

In a study conducted primarily on Caucasians, the ORs for GSTM1 and lung cancer were significant for women overall (OR = 2.50; 95% CI = 1.09-5.72) and women smokers (OR = 3.03; 95% CI = 1.09-8.40) (Tang *et al.*, 1998). Results for men including smokers showed no significant associations.

5.2.1.2 GSTT1

Like GSTM1, many studies have been conducted to determine the relationship between the GSTT1 polymorphism and possible relations to lung cancer risk and other lung diseases. Results across the studies differ moderately by showing GSTT1 null as possibly both a risk and protective factor for lung cancer. It is thought that these differences, like GSTM1 are a result of ethnicity and sample size (Ye *et al.*, 2006). Furthermore, the larger studies tend not to show much difference between the null and wild-type genotypes. A simple comparison of the meta-analysis conducted by Ye *et al.* (2006) shows that Caucasians tend to have the smallest relative risk while East Asians have the highest relative risk for the null genotype. Among cancer types, observations suggest a stronger association between squamous cell carcinoma compared to adenocarcinoma and small-cell carcinoma.

Like prior research conducted on the GSTM1 polymorphism, a majority of the large studies show little or no association with lung cancer risk (To-Figueras *et al.*, 1997; Liu *et al.*, 2001; Wang *et al.*, 2003a; Schneider *et al.*, 2004). The largest study conducted on over 1000 U.S. cases by Liu *et al.* (2001) found no association between the null genotype and lung cancer risk. Schneider *et al.* (2004) also found no overall associations between GSTT1 null and lung cancer risk among Germans. In a

population of Northwestern Mediterraneans, the GSTT1-null genotype was also not significantly associated with lung cancer risk (To-Figueras *et al.*, 1997). Similar results were also observed in Chinese patients with lung adenocarcinoma (Wang *et al.*, 2003a).

In comparison by To-Figueras *et al.* (1997), Liu *et al.* (2001), Wang *et al.* (2003a), and Schneider *et al.* (2004), several other studies have shown that GSTT1-null genotype is positively associated with an increase lung cancer risk; however, as discussed later, such findings may have been attributed to gene-gene interactions with the GSTM1-null genotype. Studies showing the highest relative risks include some of the more recent investigations by Gallegos-Arreola *et al.* (2003), Liang *et al.* (2004), and Sorensen *et al.* (2004). Gallegos-Arreoala *et al.* (2003) found a 5-fold increase in lung cancer risk among a very small group of Mexican lung cancer patients, consisting of mainly smokers. In a Chinese population, a 2-fold increase in lung cancer risk for individuals with the null genotype was observed (Liang *et al.*, 2004). These results were not only confined to non-Caucasian ethnicities as shown in a study on a population of Danes, where lung cancer risks increased 2.4-fold for individuals carrying the null genotype (Sorensen *et al.*, 2004a). It was also noted in this study that the lower age groups had the largest increase in risk and that positive associations were found for all major histological type with squamous-cell carcinoma being the highest (Sorensen *et al.*, 2004a).

Despite these findings, several studies have suggested that the null genotype may impart a decreased risk of lung cancer suggesting that the positive genotype

bioactivated a lung carcinogen. Risch *et al.* (2001) found a decreased risk of squamous cell carcinoma in German individuals carrying the null genotype. Similar results were also observed by Yang *et al.* (2004). In this study, the null genotype was associated with a decreased lung cancer risk among patients less than 50-years old (Yang *et al.*, 2004a).

When studies are broken up by smoking exposures, results also vary. Although no overall significant association was found between the GSTT1 polymorphism and lung cancer risk, Alexandrie *et al.* (2004) found that the null genotype is potentially a risk factor in Swedish light smokers, but was associated with a decreased risk in heavy smokers. In contrast, the null genotype was also found to be associated with a significant increase in cancer risk among heavy smokers in a Swedish population (Hou *et al.*, 2001). In support of these findings, among U.S. Caucasians who were heavy smokers, carriers of the null genotype had a significant increase in early-onset lung cancer (OR = 3.1; 95% CI = 1.1-8.4) (Cote *et al.*, 2005).

Among non-smoking studies, generally no associations between genotype have been observed or the non-null genotype has been associated with an increase of lung cancer risk. Among non-smoking women from the U.S., researchers found no association between the GSTT1-null genotype and lung cancer risk (Bennett *et al.*, 1999). Similar results were also observed in a study on non-smokers exposed to occupation carcinogens and indoor wood combustion (Malats *et al.*, 2000). However, Yang *et al.* (2004) found that non-smokers under the age of 50 having the GSTT1 non-null genotype had a non-significant increase risk of lung cancer (OR = 1.7; 95%

CI = 0.8 – 3.4). Although these results are not significant and exposures uncertain, they suggest an exposure to a carcinogen that is bioactivated by GSTT1.

The effect of diet on GSTT1 potential association with lung cancer risk has also been evaluated. Although the GSTT1-null genotype overall was not significantly associated with an increase in lung cancer risk, Spitz *et al.* (2000) found that the combination of the GSTT1-null genotype and low isothiocyanate intake was associated with a 3-fold (95% CI = 1.54-6.62) increase in lung cancer risk compared to individuals with the positive genotype and high intake of cruciferous vegetables. This risk was attenuated by high isothiocyanate intake. Individuals with the GSTT1 positive genotype and low dietary intake had a slight increase risk of lung cancer (OR = 1.71; 95% CI = 1.04-2.82). The tests for interactions did not meet statistical significance. In another study, no significant differences between the GSTT1 genotypes and lung cancer risk were observed in two groups with detectable and undetectable levels of isothiocyanates in urine (London *et al.*, 2000b). However, a trend of increasing risk and decreasing risk was observed in the null individuals compared to the positive individuals with undetectable and detectable levels of isothiocyanates, respectively. The relative risk among null individuals with high isothiocyanate intake was significantly lower than null individuals with undetectable isothiocyanate group (OR = 0.51; 95% CI = 0.30-0.86). No difference between the intake groups was observed in GSTT1-present individuals, suggesting that the positive genotype attenuates the anti-cancer effects of isothiocyanates.

5.2.1.3 GSTP1

In comparison to the GSTM1 and GSTT1 polymorphisms, studies on the 105V variant in the GSTP1 gene show no significant heterogeneity in regards to lung cancer risk (Ye *et al.*, 2006). While this supports the relative lack of association between GSTP1 genetic polymorphisms alone and lung cancer risk, such findings show that environmental and genetic factors may not significantly influence any associations. However, much of these findings may be attributable to the fact that the frequency of homozygous variants is very low, and as a result studies combine heterozygous and homozygous variants. Adding to this is that the activity of the GSTP1 variant relative to the wild-type is substrate-dependent. As noted earlier, the variant has higher activity toward some substrates and less activity toward others compared to the wild-type allele.

According to the latest meta-analysis by Ye *et al.* (2006) on 25 studies of the GSTP1 105V variant, the overall per allele relative risk for lung cancer was 1.04 (95% CI = 0.99-1.09). The largest study on a population of Caucasians in the U.S., consisting of mainly current smokers and former smokers, found no significant differences between cases and controls among carriers of the variant GSTP1 105 valine allele (Miller *et al.*, 2002). A lack of association between the variant GSTP1 105 allele and lung cancer risk has also been observed in other numerous studies on Caucasian populations consisting of smokers and non-smokers (Saarikoski *et al.*, 1998; To-Figueras *et al.*, 1999; Risch *et al.*, 2001; Lewis *et al.*, 2002; Perera *et al.*, 2002; Reszka *et al.*, 2003; Schneider *et al.*, 2004; Sorensen *et al.*, 2004b; Cote *et al.*,

2005). In addition, several studies on Asian populations have shown no significant associations (Kihara *et al.*, 1999; Wang *et al.*, 2003b; Chan-Yeung *et al.*, 2004; Liang *et al.*, 2004).

Despite these results, several studies have found associations between the variant and lung cancer risk. Nazar-Stewart *et al.* (2003) found a non-significant association between the GSTP1 val105 variant and risk reduction in heavy smokers. In an African-American population, Cote *et al.* (2005) observed an increase in lung cancer risk with the variant GSTP1 genotype. Observations by Stucker *et al.* (2002) and Ryberg *et al.* (1997) also suggest associations between the variant alleles and lung cancer risk. In one study, individuals homozygous for the variant GSTP1 105V genotype had a 2-fold risk (95% CI = 1.0-4.1) increase in lung cancer risk (Stucker *et al.*, 2002). Additionally, this study found that this association was mostly attributable to small-cell lung cancer. Ryberg *et al.* (1997) found a statistically significant higher incidence of the homozygous variants in male lung cancer patients compared to controls. Furthermore, mean DNA adducts in lung tissue from smokers was statistically higher in lung cancer patients with one or both of the variant alleles (Ryberg *et al.*, 1997).

5.2.2 Non-Cancer Diseases of the Lung

5.2.2.1 GSTM1

Overall, research shows that the GSTM1-null genotype is associated with decrements in lung function and the risk of developing asthma. Of particular interest is that such associations have been observed in both children and adults. Also, these

associations are modified by environment and as discussed later, gene-gene interactions.

The GSTM1-null genotype has shown to impact lung function growth in children. In a study on 4th-graders in Southern California comprised of mixed ethnicities, children carrying the null genotype with asthma had the largest statistically significant deficits in forced expiratory volume (FEV1) and forced vital capacity (FVC) (Gilliland *et al.*, 2002a). Romieu *et al.* (2004) also found an association between lung function and the GSTM1 polymorphism in children living in Mexico. In addition to measuring lung function, this study evaluated the impacts of vitamin supplements (Vitamins C and E), and accounted for exposures by measuring ambient ozone levels, PM10, nitrogen dioxide, and other climatic variables. Although the sample size was small, the placebo group ozone levels were significantly and inversely associated with the forced expiratory flow (FEF_{25-75}) in GSTM1-null genotype group. No decrement was seen in the non-null group. As for groups receiving the antioxidant vitamin supplement, the beneficial effect was observed in the GSTM1-null individuals especially in the null children having moderate and severe asthma (Romieu *et al.*, 2004). In addition to these findings, they also found that more children with moderate and severe asthma had the GSTM1-null genotype.

Recent studies have also shown that the GSTM1-null genotype may be a slight risk factor for asthma (Ivaschenko *et al.*, 2002; Kabesch *et al.*, 2004). In a northwestern Russian population of children and adults, the GSTM1-null genotype

was associated with a 3.5-fold (95% CI = 1.93–6.37) increase in asthma (Ivaschenko *et al.*, 2002), however, as will be discussed later this was due to gene-gene interactions with the GSTT1 null genotype. In German children, the GSTM1-null genotype when combined with environmental tobacco smoke (ETS), was associated with a significant increase risk of asthma (OR = 5.48; 95% CI = 1.62-18.55) and asthma symptoms compared to the wild-type with no ETS exposure (Kabesch *et al.*, 2004). Although not significant, likely due to sample size, the GSTM1 non-null genotype and ETS exposure group had an increase risk of asthma (OR = 2.94; 95% CI = 0.61-14.05). Also, the GSTM1-null genotype without ETS exposure was not significantly associated with asthma and the test for interaction did not meet significance. Therefore, it is uncertain whether the GSTM1-null genotype alone is a susceptibility factor. In contrast to these results, Holla *et al.* (2006) was unable to find an association between the null genotype and the development of allergic diseases including asthma among a population of 1000 Czechs. However, confirming previous studies by Romieu *et al.* (2004) and Gilliland *et al.* (2002a), asthmatics displayed a significant decrease in lung function, i.e., FEV1 ($p < 0.01$) (Holla *et al.*, 2006).

Despite these results, the effects of *in utero* ETS exposure cannot be ruled out as a source of these associations as several studies have shown that the combination of *in utero* exposure combined with the GSTM1-null genotype were associated with increases in asthma, on-set age, and wheeze outcomes (Gilliland *et al.*, 2002b; Kabesch *et al.*, 2004). Gililland *et al.* (2002b) found that children with GSTM1-null

genotype whom were exposed to tobacco smoke *in utero* had a significant increase risk of early onset asthma, persistent asthma, and other symptoms of asthma (i.e., wheezing). No association was found between in utero exposure and asthma risk in carriers of the GSTM1 present genotype.

In addition to asthma and lung function, other studies have been conducted on the role of this enzyme in bronchitis and chemically-induced asthma. Baranova *et al.* (Baranova *et al.*, 1997) studied the role of GSTM1-null genotypes in heavy smokers of French descent with several types of chronic bronchitis. Results of this study showed that the GSTM1-null individuals who smoked heavily were more prone to severe and moderate chronic bronchitis. They also observed that the risk of bronchitis was more prevalent in the younger age groups. Piirila *et al.* (Piirila *et al.*, 2001) also found that workers lacking the GSTM1 gene exposed to diisocyanates had a nearly 2-fold increase in diisocyanate-induced asthma.

5.2.2.2 GSTT1

Data is very limited on potential associations between the GSTT1 polymorphism and non-cancerous diseases and illnesses of the lung. Gilliland *et al.* (2002) did not find an association between the null genotype and respiratory illness in school children, whereas Ivanschenko *et al.* (2002) found a significant association between the GSTT1-null genotype and risk of asthma (OR = 6.66; 95% CI = 3.64-12.21). However, these results are likely to have been skewed due to the high frequency of GSTM1-null genotypes in the study group. Also, although several trends were not statistically significant, Kabesch *et al.* (2004) did find that the null

genotype was associated with an increase risk of asthma and all wheeze outcomes in children exposed to ETS. Of additional note, this study also found an association between *in utero* ETS exposure combined with the null genotype when evaluating lung function in children.

5.2.2.3 GSTP1

Recent research on cigarette smoke extract-induced necrosis in lung fibroblasts has suggested that GSTP1 may have a protective role in preventing smoking-related disease including emphysema (Ishii *et al.*, 2003). Similar to a model on transgenic mice that are susceptible to emphysema (D'Armiento *et al.*, 1992), Ishii *et al.* (2003) found that a decreased expression of GSTP1 led to an increase in human lung fibroblast apoptosis and necrosis, which could lead to emphysema as a result decreased production of extracellular fibrous proteins, such as collagen and elastin, or subsequent inflammation and activation of proteases (Walker *et al.*, 1988; Segura-Valdez *et al.*, 2000). Using this model and the overall expression of GSTP1 in the lung, it is postulated that the genetic variants of this enzyme could serve as a safety factor or susceptibility factor (depending on the substrate) for disease of the lung including COPD, emphysema, and asthma. As data shows, the variant GSTP1 (val105) genotype generally imparts a decrease risk of lung diseases whereas the wild-type genotype imparts an increase in disease risk. However, as with other genetic variants in the lung and other organs, such associations are likely reliant on significant exposures to chemical agents that cause these diseases as well as other genetic factors.

In regards to COPD, studies have consistently not found an association between the GSTP1 genotype and risk of COPD. No associations between GSTP1 genotype and risk of PD have been observed in populations consisting of Koreans, Taiwanese, and Chinese, Russians and Spanish (Lu and He, 2002; Yim *et al.*, 2002; Cheng *et al.*, 2004; Korytina *et al.*, 2004; Xiao *et al.*, 2004; Rodriguez *et al.*, 2005). Whereas many of these studies have evaluated smoking-related COPD, a couple of other studies have found an association between the GSTP1 Ile105 allele (wild-type) and an increase in COPD among smokers. In a population from Turkey, carriers of the homozygous and heterozygous variant GSTP1 105 alleles had significant decreases in COPD risk with ORs of 0.25 (95% CI = 0.12-0.50) and 0.47 (95% CI = 0.28-0.80), respectively, compared to the homozygous wild-type genotype (Calikoglu *et al.*, 2006). When stratified by smoking status, smokers homozygous for the wild-type allele had a significant increase in risk (OR = 3.5; 95% CI = 1-11.3). A significant association between non-smokers carrying the ile105/ile105 allele and the risk of COPD was not observed. In Japanese men, the GSTP1 ile105/ile105 genotype was associated with a statistically significant increase risk of smoking-related COPD (OR = 3.5; 95% CI = 2.7-4.6) (Ishii *et al.*, 1999).

Based on the previous findings, GSTP1 likely has little impact on the association with COPD, even in smokers. However, these observations are consistent with complexity of the disease and the notion that it may require multiple susceptible genotypes. As discussed in other sections, the GSTP1 genotype in combination with other susceptible genotypes has been significantly associated with COPD.

Unlike COPD, the association between the GSTP1 genotype and risk of asthma and bronchial hyperresponsiveness are more consistent where the variant and wild-type GSTP1 105 alleles have been associated with decreases and increases in risk, respectively. Fryer *et al.* (2000) found that the homozygous GSTP1 105 variant genotype was significantly lower in asthmatics compared to controls. This genotype conferred a 6-fold decrease in risk compared to the homozygous wild-type ($p = 0.003$). The GSTP1 105 variant was also strongly associated with bronchial hyperresponsiveness. Because ROS may modulate bronchial hyperresponsiveness, the authors suggested that the inability of GSTP1 to detoxify ROS may contribute to the development of bronchial hyperresponsiveness and asthma (Fryer *et al.*, 2000). Confirming these findings, Taiwanese school children carrying the GSTP1- Ile105 allele had a significant increase in risk of physician-diagnosed asthma (Lee *et al.*, 2005b).

Similar to other associations between genotype and disease risk, the levels of exposure to xenobiotics may also determine whether the variant allele confers a decrease in asthma. In Taiwanese school children, Lee *et al.* (Lee *et al.*, 2004) found that individuals homozygous for the GSTP1 wild-type had a significantly increased risk of asthma in high air pollution. In high air pollution alone, the increase in risk for the individuals homozygous for the wild-type 105 allele compared to individuals carrying the variant 105 allele was nearly 4-fold ($p < 0.05$). However, this difference was not observed between the genotypes in low or moderate air pollution, suggesting that the risk of asthma revealed a dose-response relationship with outdoor air

pollution in children homozygous for the variant allele (Figure 12). The test for interaction was statistically significant ($p = 0.035$).

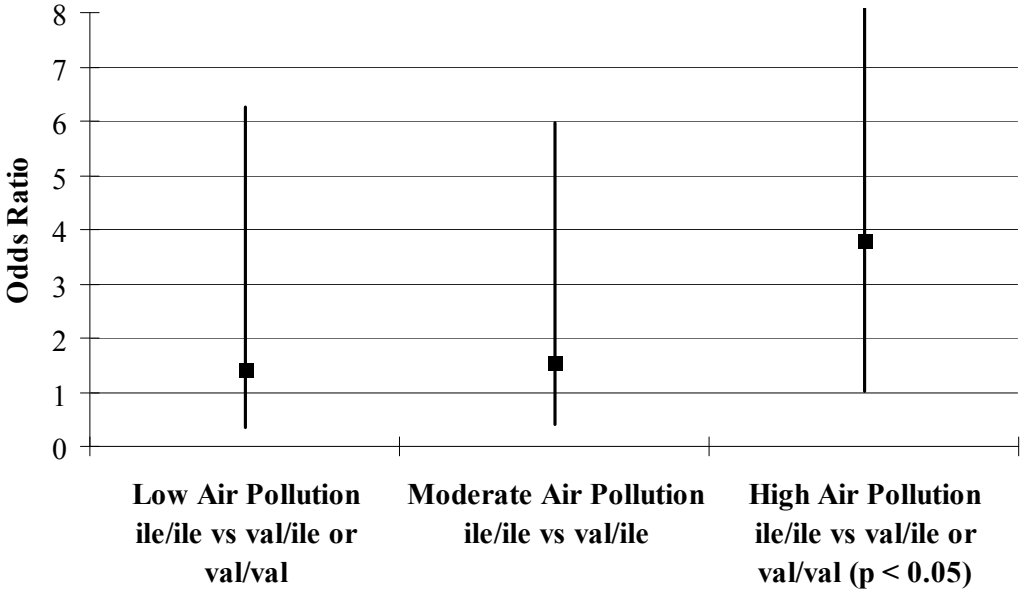

Figure 12. Relative Risk of Asthma for GSTP1 105 wild-type versus 105 variant in Low, Moderate, and High Air Pollution. Based on data from Lee *et al.* (2004)

The impact of xenobiotic exposure levels on associations between genotype and asthma has also been observed in occupational related asthma due to exposure to toluene diisocyanate (TDI), a reactive electrophile that covalently binds to proteins producing immunogenic proteins. This results in airway inflammation, oxidative stress, and ROS generation. Consistent with other studies linking the wild-type GSTP1 allele and increase risk of asthma, Mapp *et al.* (2002) found that the homozygous GSTP1 variant genotype was lower in individuals who had TDI-induced asthma. However, these observations were only made in those who were exposed to

TDI for more than ten years. In addition, the protective effect of the variant allele increased in proportion to duration of exposure (Mapp *et al.*, 2002).

Despite the previous findings, it is important to consider that the association between the GSTP1 genotype and risk of lung diseases may also be dependent on substrate. This may explain several studies where the variant GSTP1 105 allele was associated with an increase in asthma severity and decline in lung function (Gilliland *et al.*, 2002a; Ercan *et al.*, 2006). In children with upper and low respiratory illness, Gilliland *et al.* (2002a) found that the GSTP1 105 variant allele was associated with slower lung function growth compared to the wild-type alleles. They suggested that the variant allele is less able to defend against products of oxidative stress mediated by viral infections. A decreased ability to defend against products of oxidative stress may explain why the GSTP1 val/val genotype has been significantly associated with asthma severity in Turkish children (OR = 4.2; 95% CI = 1.6-11.2) (Ercan *et al.*, 2006). Assuming the variant has a decreased ability to detoxify products of oxidative stress, it is possible that the GSTP1 wild-type 105 allele's association with increase risk of COPD and asthma risk discussed previously may be independent of ROS, or there is differential activity towards different products of oxidative stress. Perhaps these findings are due to chance, where these genotypes are in linkage disequilibrium with other susceptible genotypes that actually contribute to the increased risks of COPD and asthma.

5.2.3 GSTs and Gene-Gene Interactions in Lung Diseases

Although research shows there is slight association between individual GST genotypes and risk of lung cancer and other lung diseases, gene-gene interactions significantly modify the association between GSTs and risk of lung disease. These interactions may occur between the GSTs or between individual GSTs and genes of other enzymes (e.g., P450s, mEH). Also, as will be shown, gene-gene interactions may have contributed to the associations observed between single genes and disease risk.

5.2.3.1 Interactions between GSTs

Some of the best examples of gene-gene interactions are found between the GSTM1, GSTT1 and GSTP1, which is somewhat expected given they share some common substrates. Overall, data suggests a weak interaction between the GSTT1-null genotype and either of the susceptible GSTP1 or GSTM1 genotypes, but that the interactions may be stronger according to the level exposure, ethnicity, and type of disease. Also, interactions between the GSTP1 and GSTM1 genotypes and an association with increase in lung cancer have been observed, which is supported by findings of increases in DNA adduct levels among individuals carrying both the GSTM1-null genotype and variant GSTP1 genotype (Ryberg *et al.*, 1997).

When the individuals are concurrently lacking both the GSTM1 and GSTT1 genes, increases in lung cancer risk have generally not been observed (To-Figueras *et al.*, 1997; London *et al.*, 2000b; Stucker *et al.*, 2000; Nazar-Stewart *et al.*, 2003; Cote *et al.*, 2005). However, lung cancer risk has shown to be significantly increased in

other studies with ORs ranging from 2.1 to 2.9 ($p < 0.05$) (Kelsey *et al.*, 1997; Saarikoski *et al.*, 1998; Sorensen *et al.*, 2004a). Of interest is that Sorenson *et al.* (2004a) found that the GSTT1-null genotype alone was associated with lung cancer risk, found that when GSTM1-null and GSTT1-null genotypes were combined, only the GSTT1-null genotype was associated with lung cancer risk in individuals carrying the GSTM1-null genotype or 1 or 2 GSTP1 variant alleles (Figure 13).

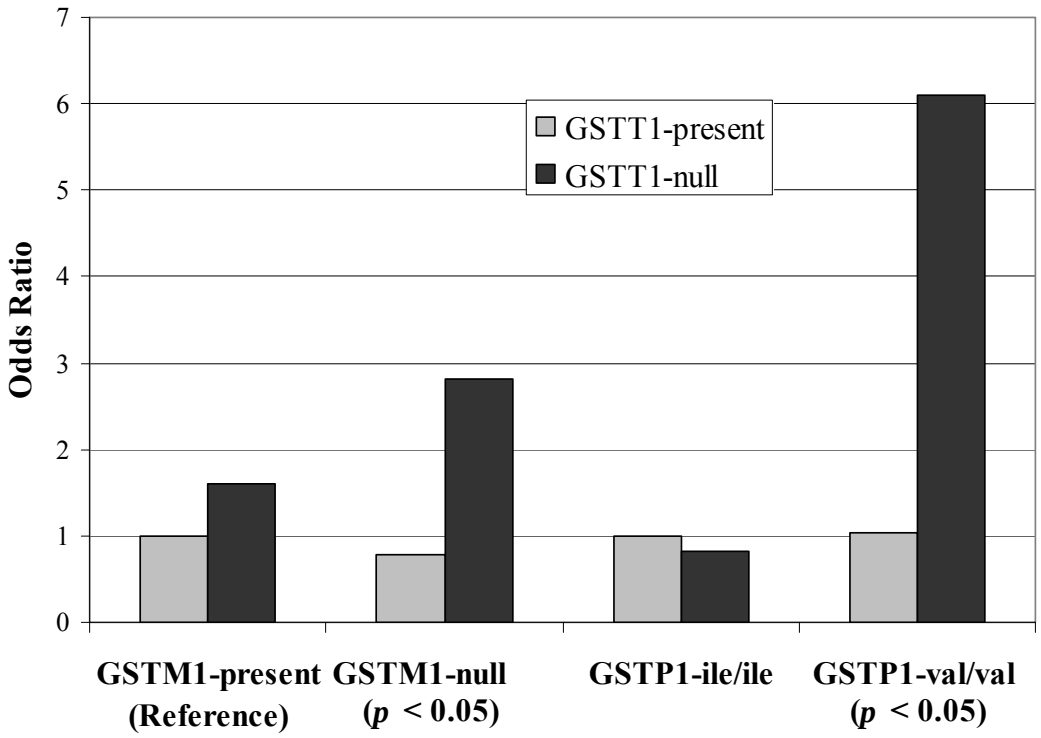

Figure 13. Lung Cancer Odds Ratios for Combinations of GSTs. Based on data from Sorenson *et al.* (2004a)

Upon further review of these studies, a combination of environmental interactions and ethnicity may play a role in these discrepancies. For example, Cote

et al. (2005) found that the combination of the GSTM1-null and GSTT1-null genotypes was only significantly associated with an increase in lung cancer among Caucasian heavy smokers (OR = 5.0; 95% CI = 1.1-23.6). No associations between the combination of the null genotypes and lung-cancer risk were observed in the light smoking group or among African-Americans.

While concurrent lack of both GSTM1 and GSTT1 may not generally impart an increase risk of lung cancer, due to expression levels and substrate specificity (with regards to carcinogens), significant increases in risk of asthma have been observed. This is consistent with findings that have demonstrated these enzymes share activity toward products of oxidative stress, such as phospholipid hydroperoxide, that facilitate the production of pro-inflammatory eicosanoids (Hayes and Strange, 1995; Strange and Fryer, 1999). Ivaschenko *et al.* (2002) found that the concurrent lack of GSTM1 and GSTT1 was associated with an 8.5-fold (95% CI = 3.6-19.9) increase risk of asthma among Russians. Interestingly, this study also found that alone these genotypes were associated with a significant increase in asthma risk, but similar to the Sorenson *et al.* (2004) study, the impact of the gene-gene interaction likely contributed to a majority of the increase risk among genotypes alone. Approximately half (54%) of the cases were lacking both genes. A similar increase in risk was also observed by Saadat *et al.* (Saadat *et al.*, 2004), with a 10.2-fold (95% CI = 3.58-29.29; $p < 0.00001$) increase in asthma risk. When combined with the susceptible CC16 gene, which encodes a protein involved in inflammation and

immune modulation, risk increased 22-fold (95% CI = 4.08-123.48; p = .00004) (Figure 14).

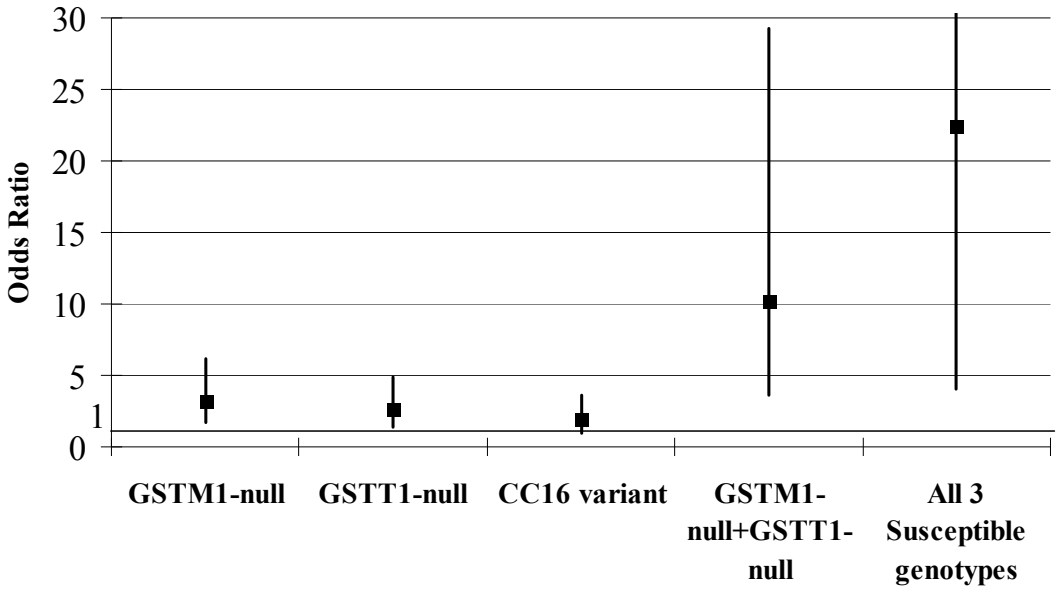

Figure 14. Relative Risk of Asthma for GSTM1, GSTT1, and CC16 genotypes. Based on data from Saadat *et al.* (2004)

Similar associations have also been observed in other studies (Tamer *et al.*, 2004; Zhang *et al.*, 2004). However, a large study in a Czech population found no significant association between the combined genotype and increase risk of atopic asthma.

In regards to GSTM1 and GSTP1, the interaction of susceptible genotypes has also been implicated in increasing the risk of lung cancer. The GSTM1-null and GSTP1 val105 allele in combination yielded a significant 2.4-fold (95% CI = 1.1-5.1) increase risk of adenocarcinoma among a Chinese population of non-smokers and smokers (Wang *et al.*, 2003). No significant associations with lung cancer were

found in the genotypes alone. Similar results have also been observed in Caucasians (Miller *et al.*, 2002; Perera *et al.*, 2002). However, the latter study by Miller *et al.* (2002) only found the association to be significant in early-onset cancer (OR = 4.03; 95% CI = 1.47–11.08; $p < 0.01$). When considering different histology types, the combination of the GSTM1-null and GSTP1 variant alleles have also been associated with an increase in risk for squamous cell carcinoma, adenocarcinoma, and small-cell carcinoma in male smokers aged 50-69 years of age (Kihara *et al.*, 1999) (Figure 15).

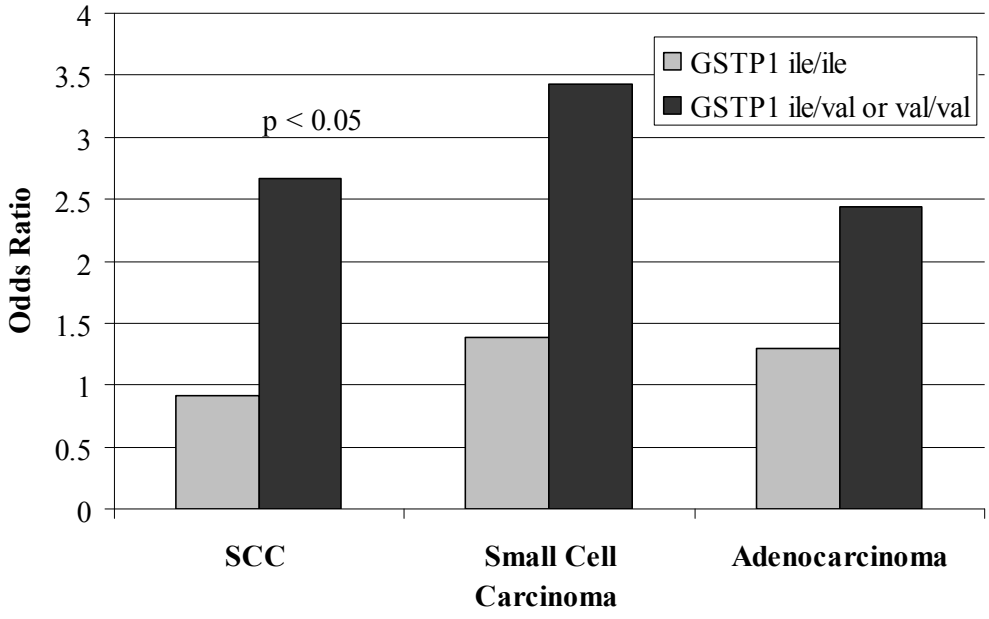

Figure 15. Relative Risk for GSTM1-null for Lung Cancer according to GSTP1 genotype. Based on data from Kihara *et al.* (1999)

In addition to lung cancer, the variant GSTM1 and GSTP1 genotypes have been associated with increases in risk of COPD. As noted earlier, the GSTP1 wild-

type combined with the GSTM1-null and slow mEH genotypes were associated with a significant increase in COPD (Cheng *et al.*, 2004).

5.2.3.2 Gene-Gene Interactions with Other Genotypes

In addition to gene-gene interactions between GST isozymes, it is also important to consider interactions between GSTs and other biotransformation enzymes. First, GSTs genotypes may interact with other biotransformation enzymes that compete for the same substrate (i.e., mEH and NQO1), which is discussed in other sections. Also, GSTs may interact with other genotypes at different steps within a particular biotransformation pathway. GSTs often detoxify reactive intermediates produced by other biotransformation reactions. Therefore, altered activity of these enzymes that produce reactive intermediates may affect the overall association between the susceptible GST genotypes and risk of lung disease and vice versa.

Genotypes that may interact with GST genotypes include, but are not limited to, genes encoding Cytochrome P450 1A1 (CYP1A1) and *N*-acetyltransferase 2 (NAT2). Primary substrates for CYP1A1 include large polyaromatic hydrocarbons, such as benzo(a)pyrene, whereas the NAT2 enzyme is responsible for the catalysis of amines, including arylamines and heterocyclic amines (Wormhoudt *et al.*, 1999). Polymorphisms have been observed in these enzymes and have been well studied. As mentioned previously, polymorphisms within the CYP1A1 gene may confer increased enzyme activity (Wormhoudt *et al.*, 1999). Mutations within the NAT2 enzyme may confer decreased enzymatic activity (i.e., "slow acetylator"). Given

polymorphisms within these genes could affect enzyme activity, a greater amount of the DNA-reactive products can be produced.

Numerous studies have found gene-gene interactions between GSTs and CYP1A1 that have contributed to an increase in lung cancer risk. First, the GSTM1-null genotype combined with the CYP1A1 variants conferring greater activity have been associated with significant increases in DNA adduct levels (Butkiewicz *et al.*, 1999; Alexandrov *et al.*, 2002). When looking at associations with lung cancer risk, similar results are observed for these genotypes. In a pooled analyses of 14 studies on mostly Caucasian non-smokers from Europe and the U.S., Hung *et al.* (Hung *et al.*, 2003) found that the CYP1A1 Val 462 and Msp1 variants were associated with an increase in lung cancer risk; however, when grouped according to GSTM1 genotype, increases in risk were only observed in GSTM1-null individuals. The combination of the CYP1A1 Val442 variant and GSTM1-null genotypes were associated with a significant increase in adenocarcinoma risk (OR = 4.67; 95% CI = 2-10.9) compared to CYP1A1 Ile442 wild-type + GSTM1-present genotypes. A non-significant increase (OR = 2.44; CI = 0.94-6.33) for the GSTM1-null and CYP1A1 Msp1 variant was also observed in the pooled analysis (Figure 16).

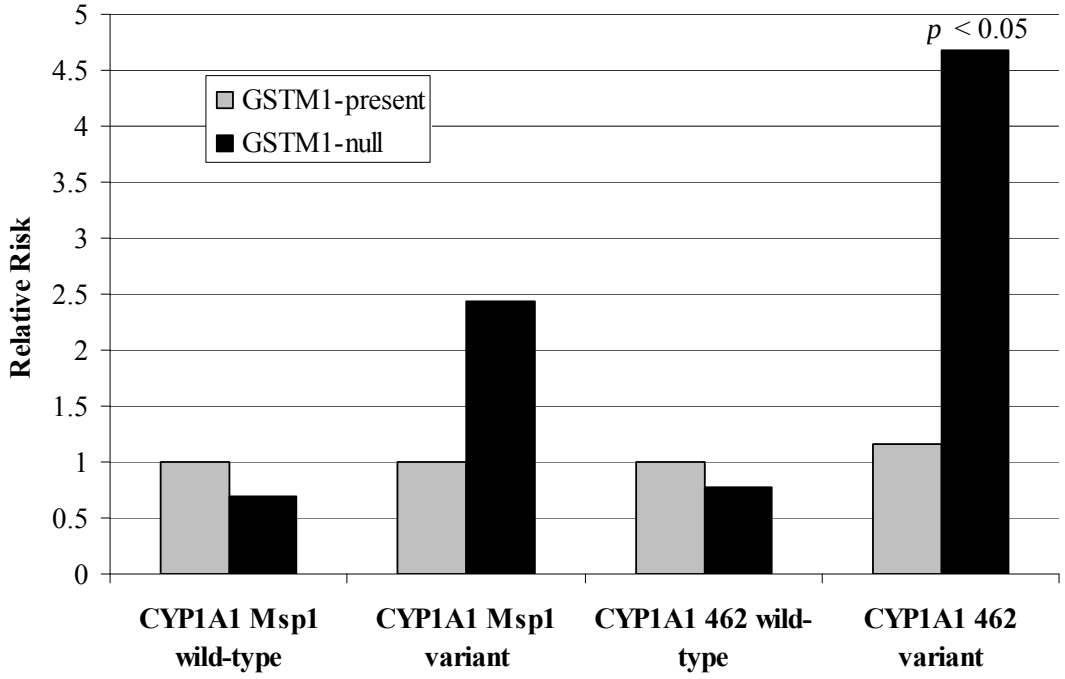

Figure 16. Relative Risk for Combinations of GSTM1 and CYP1A1 genotypes among Caucasian Non-Smokers. Based on data from Hung *et al.* (2003)

Although the combination of the GSTM1-null genotype and CYP1A1 variants may be associated with lung cancer risk, smoking likely modifies this risk and is more significant in smokers. Contrary to findings by Hung *et al.* (2003), a recent pooled-analysis of non-smokers found no significant association between the combination of the GSTM1-null and CYP1A1 genotypes and increase in lung cancer risk (Raimondi *et al.*, 2005). However, studies on smoking have found significant associations between the combined GSTM1-null and CYP1A1 variant genotypes and lung cancer (Kihara *et al.*, 1995; Le Marchand *et al.*, 1998; Quinones *et al.*, 2001; Sobti *et al.*, 2004; Yang *et al.*, 2004b; Sreeja *et al.*, 2005). In support of an interactive role, Le Marchand *et al.* (1998) found that an increase in squamous cell carcinoma

risk was only observed when the CYP1A1-Msp1 homozygous variant (m2/m2) was combined with the GSTM1-null genotype (OR = 3.1; 95% CI = 1.2-7.9) (Figure 17). However, interaction between the two genotypes did not meet statistical significance.

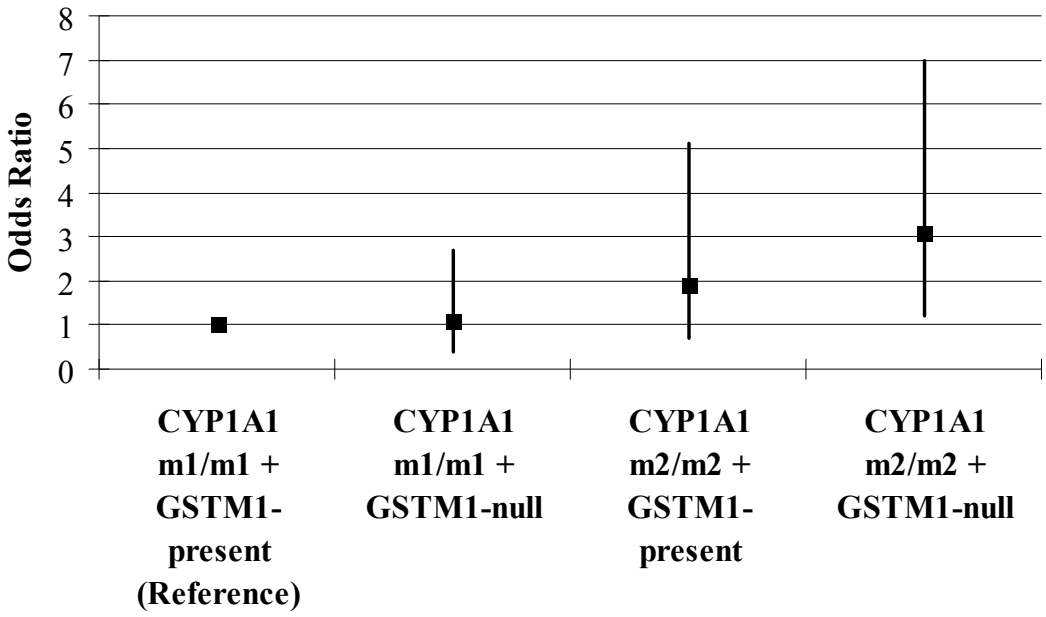

Figure 17. Relative Risks of Squamous Cell Carcinoma for Combinations of CYP1A1 and GSTM1 genotypes. Based on data from Le Marchand *et al.* (1998).

Among Japanese smokers, the CYP1A1 Msp1-homozygous variant combined with the GSTM1-null genotype has also been associated with an increase in lung cancer risk, but when evaluated separately, neither susceptible genotype was associated with a significant increase in lung cancer risk (Kihara *et al.*, 1995).

Associations between the combinations of at-risk genotypes of GSTM1 and CYP1A1 have also been observed in areas of high pollution. In a polluted region in

Poland, individuals carrying both the GSTM1-null and CYP1A1 variant genotypes had a significant increase in risk of adenocarcinoma (Butkiewicz *et al.*, 1999).

In additional to interactions between CYP1A1 and GSTs, gene-gene interactions may also occur between GSTs and NATs. In Swedish smokers, individuals carrying a combination of the GSTM1-null and NAT2-rapid genotypes showed associations with DNA-adduct levels and HPRT-mutant frequency (Hou *et al.*, 2001). Hou *et al.* (2000) found that the GSTM1-null and NAT-slow genotypes increased in non-operable lung cancer among Norwegians, including squamous cell carcinoma at young age and low dose to cigarettes. A similar association between diisocyanate induced asthma was also observed in carriers of both the GSTM1 null genotype and slow NAT1 (OR = 4.53; 95% CI = 1.76 – 11.6) and NAT2 genotypes (OR = 3.12; 95% CI = 1.11 – 8.78) (Wikman *et al.*, 2002). Despite these results Nyberg *et al.* (Nyberg *et al.*, 1998) found that non-smokers having the GSTM1-positive and NAT-slow genotypes were associated with an increase in lung cancer (OR = 3.1; 95% CI = 1.1 – 8.6). No associations were found in smokers or the entire study group.

5.2.3.3 Gene-Gene and Gene-Environment Interactions

While the combination of the susceptible GST genotypes may be associated with increases in cancer risk, environmental factors, especially smoking (i.e., intensity) may enhance these gene-gene interactions. For example, Cote *et al.* (2005) found no significant associations between combinations of susceptible genotypes and early onset cancer among a group of Caucasian smokers and non-smokers. However,

when stratified according to smoking status, the study found a significant trend in increasing risk among the heavy smokers as the number of susceptible genotypes increased. The ORs for 1, 2, and 3 susceptible genotypes among heavy smokers was 1.3 (95% CI = 0.5 -3.1), 2.1 (95% CI = 0.8 – 5.4) and 7.0 (95% CI = 0.8 – 62.4) with a p-value of 0.03 for the trend (Figure 18). No trends were observed in the light smoking or non-smoking groups (Cote *et al.*, 2005).

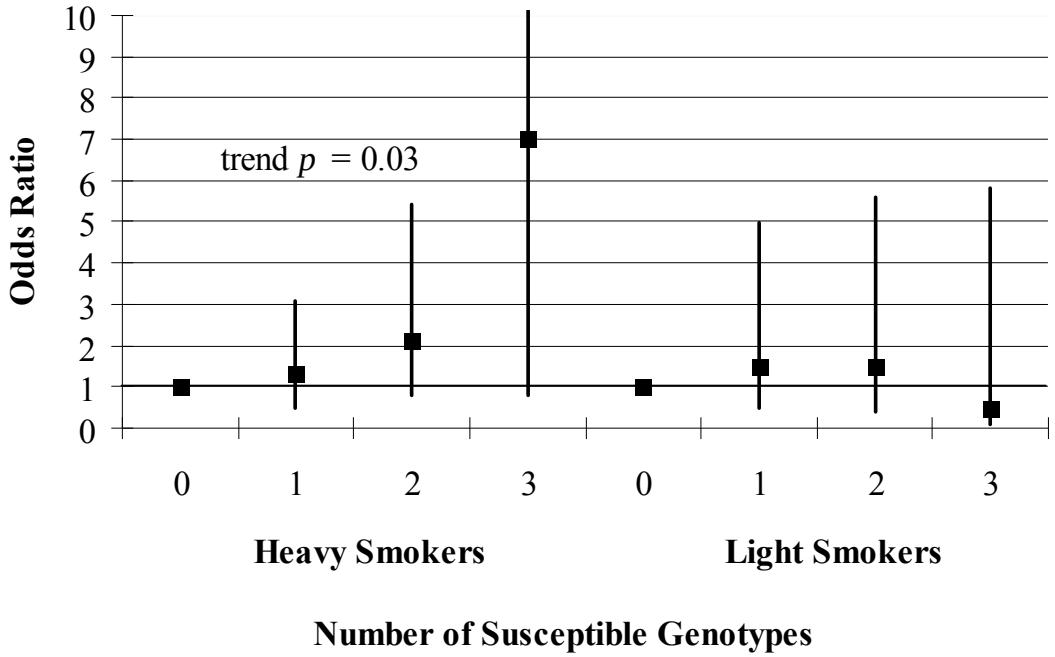

Figure 18. Relative Risks for Early-Onset Lung Cancer in Caucasians with Increasing Numbers of GST Susceptible Genotypes. Based on data from Cote *et al.* (2005).

5.3 Bladder Cancer

Urine among smokers with variant forms of GSTs is more mutagenic compared to that of smokers with wild-type GSTs (Hirvonen *et al.*, 1994; Alexandrie

et al., 2000). Additionally, and of greater importance are findings that GST genotypes may play a role in the development of bladder cancer. Similar to the lung, associations between GSTs and bladder cancer risks are modified and/or dependent on other factors including the types and levels of exposure to bladder carcinogens, gender, ethnicity, diet, and genetic interactions with other biotransformation enzymes, repair mechanisms, etc.

5.3.1 Potential mechanisms of bladder carcinogenesis.

There is significant uncertainty regarding the exact roles the variant forms of the GST isozymes may play in providing protection or susceptibility to bladder cancer. GSTs may detoxify reactive metabolites that contribute to the bladder carcinogenesis (e.g. PAH diol epoxides, benzidine and other arylamines) and they may also detoxify products of oxidative stress, such as cytotoxic lipids (Hayes and Strange, 1995). Also, conjugation may lead to reactive metabolites (e.g., thiol-derivatives). Of course these roles will be dependent upon the substrate, but having the variant form (null in the case of the GSTM1 and GSTT1 isozymes) may afford greater or less protection whether it is in a detoxifying or bioactivating role.

In the context of biomarkers, several studies have been conducted on urine levels of 1-hydrooxypyrene (1-OHP), a metabolite of PAH metabolism, to determine whether GST polymorphisms affect biomarkers of exposures. Such information could provide insight on the extent of susceptibility to carcinogenic PAHs (Alexandrie *et al.*, 2000; Yang *et al.*, 2003). Note, GST activity, especially GSTM1, is not directly linked to metabolism of 1-OHP; however, GST activity could impact

the glucuronidation pathway and increase or decrease the formation of the 1-OHP glucuronide (Alexandrie *et al.*, 2000). In these studies, levels of 1-OHP were measured in urine of individuals exposed to PAHs in the workplace (coke ovens, aluminum production plant) as well as individuals who were not exposed to PAHs in the workplace (Merlo *et al.*, 1998; Pan *et al.*, 1998; Alexandrie *et al.*, 2000; Zhang *et al.*, 2001; Yang *et al.*, 2003). In studies on coke oven workers, the GSTM1 genotype has generally not been found to be associated with increased levels of 1-OHP. This may be explained due to a lack of activity whether indirectly or directly with 1-OHP, or that high exposures mask any association by saturating enzymatic systems. Additionally, in support of gene-gene interactions, one study found that when the GSTM1-null genotype was combined with variant CYP1A1 allele, urinary 1-OHP levels were substantially higher, compared to other genotype combinations (Alexandrie *et al.*, 2000). Despite these findings, no association was found among GSTM1 polymorphisms and urinary levels of 1-OHP in coke oven workers in China and police officers in Italy (Merlo *et al.*, 1998; Pan *et al.*, 1998). In regards to GSTT1 and GSTP1, none of these studies found any significant associations between the genotypes and 1-OHP urine levels.

Assuming GSTs are linked to 1-OHP, the lack of association may be a result of a saturation of enzymatic activity. Somewhat in support of this theory, is a study conducted on a Korean population whom were not exposed to occupational PAHs, found that GST polymorphisms impacted urinary levels of 1-OHP (Yang *et al.*, 2003). When evaluating genotypes independently, Yang *et al.* (2003) found that the

GSTT1 present genotype had a significantly higher level of urinary 1-OHP. While GSTM1 alone did not affect 1-OHP levels, the GSTM1-null genotype did increase levels of 1-OHP when combined with GSTT1 present genotype (Yang *et al.*, 2003). Although such findings do not indicate toxicity, they are consistent with the mechanisms by which GSTT1-present and GSTM1-null genotypes are associated with bladder cancer risk. As will be discussed later, the GSTT1-present genotype may be associated with an increase in cancer risk via bioactivation of carcinogens.

Previous studies have also reported a mutation in the *p53* tumor suppressor gene in urinary bladder cancer (Sidransky *et al.*, 1991; Spruck *et al.*, 1993; Williamson *et al.*, 1994; Brockmoller *et al.*, 1996b; Martone *et al.*, 1998). In addition, p53 mutations have been associated with susceptible genotypes (Martone *et al.*, 1998; Ryk *et al.*, 2005). In a study on Swedish bladder cancer patients, Ryk *et al.* (2005) investigated GST polymorphisms as they related to *p53* mutational status. They found that although not significant, GSTT1 and GSTM1-null genotypes were overrepresented among patients with the *p53* mutation, compared to patients without the mutation. These observations were not observed for the GSTP1 genotypes. When looking at the mutation subtypes (transversions/transitions), Ryk et al (2005) did find a significantly higher proportion of transversions in the GSTM1-null individuals and smokers carrying at least 1 GSTP1 variant allele. Because transversions are more related to exogenous exposure than transitions, these observations may be a result of less efficient detoxification of exogenous compounds (Martone *et al.*, 2000; Ryk *et al.*, 2005). Ryk *et al.* (2005) suggested that the GSTP1 val105 allele may have been

less efficient in detoxifying carcinogens to which study group may have been exposed.

Martone *et al.* (2000) observed the opposite among Italian patients. In this study, *p53* mutations was found to be 3.5-fold greater ($p = 0.03$) in individuals with the ile105/ile105 genotype compared to the variant. No associations were found between the GSTM1 and GSTT1 genotypes and *p53* mutations. The authors suggested GSTP1 involvement in the activation of unknown carcinogens derived from tobacco smoke (e.g. quinones or organic halides) and/or inactivation via conjugation of prostaglandin A2 and J2, which are inhibitors of cell proliferation (Atsmon *et al.*, 1990; Sato *et al.*, 1990; Bogaards *et al.*, 1997; Martone *et al.*, 2000).

In addition to possible associations with *p53* mutations, GSTs have been associated with increased damage to urothelial cells. Lebrun *et al.* (2006) carried out studies to investigate any possible associations between GST genotypes and ochratoxin A toxicity in primary human urothelial cells. The mycotoxin ochratoxin A is a food contaminant found in many human foods including but not limited to grains, breads, nuts, coffee, and animal meat (Lebrun *et al.*, 2006). Ochratoxin A is known to be genotoxic, immunotoxic, teratogenic, and carcinogenic in rodents, and is classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC, 1993). Ochratoxin A has also been linked to urinary tract tumors (Lebrun *et al.*, 2006). Although the exact mechanism of toxicity is not certain, several potential mechanisms have been proposed including toxicity of the Ochratoxin A molecule itself, formation of an ochratoxin quinone and hydroquinone

resulting in the formation of reactive oxygen species, formation of a ochratoxin radical by a peroxidase, and a thioderivative formed from the ochratoxin glutathione conjugate. Upon data evaluation, Lebrun *et al.* (2006) found that the GSTT1-present, GSTM1-null, and variant GSTP1 genotypes were found more frequently in the damaged urothelial cells. Although the author expressed caution with their findings given distribution of the GSTT1 genotype in Caucasians and the small number of samples, the GSTT1 results support a role of bioactivation. As for the GSTM1 and GSTP1 results, the data supports a role of detoxification of ochratoxin toxicity.

5.3.2 GSTM1

The GSTM1 isozyme has been consistently shown to be associated with bladder cancer risk among different ethnicities including Europeans, Asians, and Americans (Bell *et al.*, 1993; Daly *et al.*, 1993; Brockmoller *et al.*, 1996a; Kempkes *et al.*, 1996; Georgiou *et al.*, 2000; Giannakopoulos *et al.*, 2002; Lee *et al.*, 2002). These studies show that the null genotype is associated with a modest increase in bladder cancer risk with statistically significant associations, having ORs ranging from 1.6 to 3.8 (Daly *et al.*, 1993; Lee *et al.*, 2002). Three meta-analyses conducted by Johns and Houlston (Johns and Houlston, 2000), Engel *et al.* (2002), and Garcia-Closas *et al.* (2005) have found statistically significant associations between the GSTM1-null genotype and bladder cancer risk with ORs of 1.5, 1.4, and 1.5, respectively. As expected, such associations are generally observed in studies on individuals exposed to significant levels of bladder carcinogens whether it is through smoking and/or occupational exposures to arylamines, both of which have been

linked to bladder cancer and whose metabolic products are substrates for GSTs (Case and Pearson, 1954; Cohen, 1981; Silverman *et al.*, 1992). Alternatively, it has been observed that GSTM1 genotype association with bladder cancer is independent of the severity of exposure, which supports the idea that the effects of genetic traits may be overwhelmed by the saturation of enzymatic capacity at high doses (Hietanen *et al.*, 1997; Perera, 1997; Filiadis and Hrouda, 2000).

Several studies have been conducted showing that the association between the GSTM1 and bladder cancer risk are dependent on exposure to relatively high levels of bladder carcinogens. When stratifying results according to smoking status, the GSTM1- null genotype tends to be associated with an increase in bladder cancer risk compared to GSTM1-present genotype among smokers, whereas little or no association is found in non-smokers (Bell *et al.*, 1993; Katoh *et al.*, 1998; Salagovic *et al.*, 1998; Salagovic *et al.*, 1999; Toruner *et al.*, 2001; Lee *et al.*, 2002; Jong Jeong *et al.*, 2003; Hung *et al.*, 2004b; Moore *et al.*, 2004) (Figure 19).

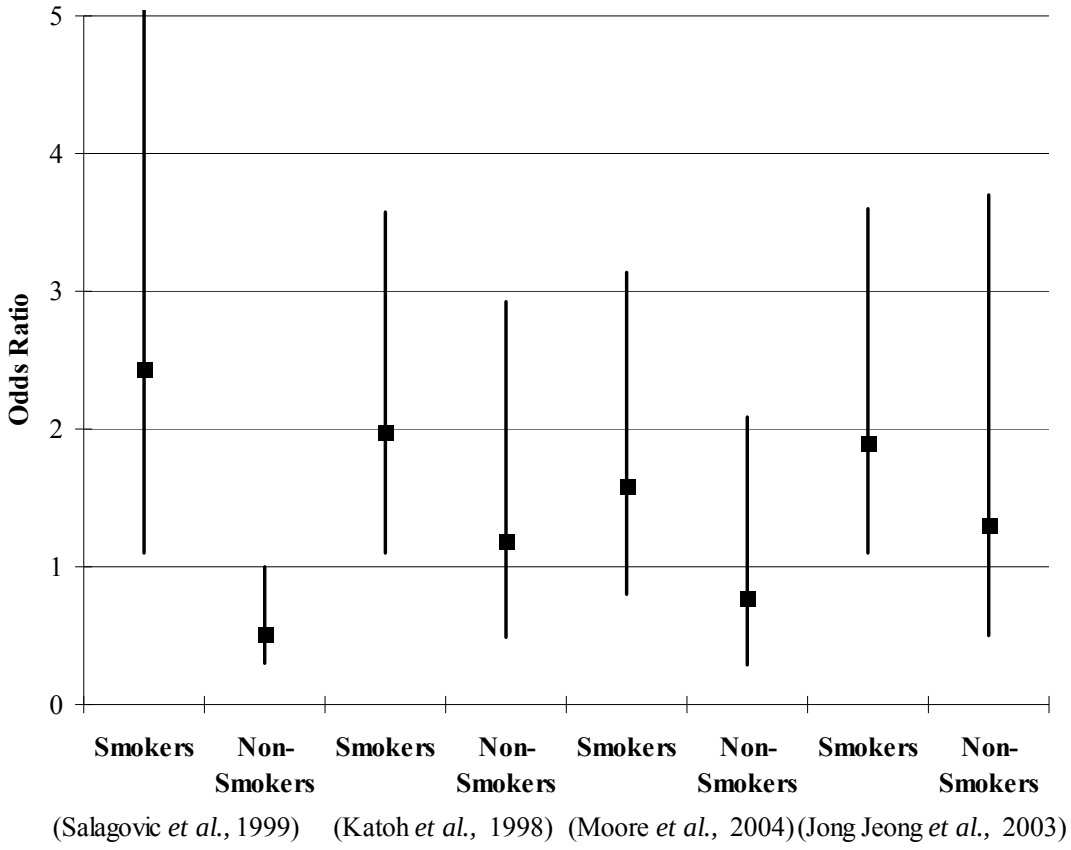

Figure 19. Relative Risk of Bladder Cancer for Smokers and Non-Smokers for GSTM1-null Genotype Compared to GSTM1-present Genotype

The ORs among statistically significant associations range from 1.8 to 2.44 (Bell *et al.*, 1993; Salagovic *et al.*, 1999). Additionally, as exposure to tobacco smoke increases, bladder cancer risk among null genotypes may increase synergistically (Hung *et al.*, 2004b). However, synergistic interactions were not observed by Engel *et al.* (2002) and Garcia-Closas *et al.* (2005) in pooled analyses. Rather, the interactions may be additive (Engel *et al.*, 2002).

In addition to GSTM1's association with bladder cancer risk in the context of smoking related exposures, the genotype may also associated with increased bladder cancer risk among non-smoking related exposures to bladder carcinogens (including unidentified bladder carcinogens). Similar to smoking, research suggests that the effect of the null genotype on bladder cancer requires significant exposures to carcinogens, such as occupational exposures, rather than typical environmental exposures. This is supported in a study on German bladder cancer patients in an area containing coal, iron, and steel industries (Golka *et al.*, 1997). In this study, the null genotype was overrepresented in bladder cancer cases for workers exposed to arylamines and PAHs on the job, including exposures to colorants, coke oven emissions, and coal (Golka *et al.*, 1997). However, the distribution of the null genotype was normal in bladder cancer cases among businessmen and administrative officers. Consistent with the need for significant exposures to bladder carcinogens, Kim *et al.* (2000) found that the null genotype was significantly associated with bladder cancer patients who had a history of asthma or tuberculosis. Although the mechanisms and responsible agents are unknown, it was suggested that the null-allele subjects may have a decreased ability to detoxify reactive intermediates of drugs used to treat the two diseases (Kim *et al.*, 2000). Interestingly, this study found no association between the GSTM1-null genotype and bladder cancer risk among smokers.

While the effect of the null genotype on bladder cancer seems to rely on significant exposures to bladder carcinogens, findings from other studies are to the

contrary. Several studies conducted on bladder cancer patients from North India, United Kingdom, Spain, Germany, Netherlands, and U.S., have shown that the association between the GSTM1 genotype is independent of significant exposures to bladder carcinogens (Brockmoller *et al.*, 1994; Kempkes *et al.*, 1996; Mungan *et al.*, 2000; Srivastava *et al.*, 2004b; Garcia-Closas *et al.*, 2005). Several of these studies have found modest statistically significant increases in bladder cancer risk among non-smokers carrying the null genotype, similar to that observed in studies showing smoking as a dependent factor. When looking into occupational exposures to bladder carcinogens, Brockmoller *et al.* (1994) found no significant difference in GSTM1 genotypes between individuals with at-risk jobs versus those without.

In addition to the impacts of smoking on the association between GSTM1 and bladder cancer risk, several studies have evaluated the impact of gender on this potential association. As found in numerous studies, bladder cancer is consistently higher in men; however, this may be due to historical differences in occupational exposures and smoking habits (Engel *et al.*, 2002; Srivastava *et al.*, 2005). Regardless, research on associations between GSTM1 and bladder cancer have looked into the impacts of gender. In fact, gender may explain some of the differences observed among the previously mentioned studies. Among women in North India, a nearly significant association was found between the GSTM1-null genotype and bladder cancer risk (OR = 3.7; $p = 0.054$), but no association was found among men (Srivastava *et al.*, 2004b). Karagas *et al.* (2005) also found that the null genotype was significantly associated with bladder cancer risk among female smokers

(OR = 2.3; 95% CI = 1.1-4.5), but not male smokers. In contrast to these studies, a significant association (OR = 3.23; 95% CI = 1.38-7.58) was found between the null genotype and bladder cancer risk among male non-smokers (McGrath *et al.*, 2006). No such association was found among female smokers, even after adjusting for smoking status.

Whereas the previous results provide no clear pattern, they do show that gender difference among particular populations can impact the associations between GSTM1 and bladder cancer. Gender differences could range from exposures to greater levels of bladder carcinogens in one gender to gender difference (i.e., genetic susceptibility) that is unique to a particular region or ethnicity.

In regards to the potential impacts of ethnicity or geographic regions, no significant differences have been observed, albeit a majority of the studies are confined to studies on Europeans and Caucasians. When pooling data according to geographic region, adjusted ORs for Asians, Europeans, and the U.S. were 1.7 (95% CI = 1.19-2.42), 1.29 (95% CI = 1.05-1.58), and 1.49 (95% CI = 1.06-2.08), respectively (Engel *et al.*, 2002). In smokers, the ORs for individuals from Europe and U.S. were 1.73 (95% CI = 1.35-2.20) and 3.34 (95% CI = 2.18-5.11). An OR for Asians could not be estimated due to lack of studies.

5.3.3 GSTT1

Overall, available data suggests that GSTT1 is not independently associated with bladder cancer risk (Garcia-Closas *et al.*, 2005). This is consistent with the fact that GSTT1 has little or no activity toward known carcinogens in tobacco smoke,

such as PAHs (Srivastava *et al.*, 2004b). Rather, GSTT1 is strongly involved in the metabolism of low-molecular-weight electrophiles, such as monohalomethanes and ethylene oxides that are not considered to be bladder carcinogens (Guengerich *et al.*, 1995). Despite these current understandings regarding GSTT1s role in detoxification and bioactivation, results in available studies do vary, but do show that GSTTT1 is not independently associated with bladder cancer risk. Such inconsistencies may be a result of the types of studies conducted, which often evaluate the impacts of smoking on bladder cancer risk. Also, GSTT1 may be involved in the detoxification or toxification of unknown exogenous or endogenous chemicals that have not been identified (Filiadis and Hrouda, 2000). Complicating this potential role are modifying factors such as ethnicity and gender, as well as potential interactions with other enzymatic systems including but not limited to other GSTs, CYPs, and NATs. In fact, any role the GSTT1 polymorphism may play in the susceptibility to bladder cancer may rely on gene-gene interactions.

Generally, no association between the GSTT1 genotype and bladder cancer risk has been observed (Georgiou *et al.*, 2000; Kim *et al.*, 2000; Steinhoff *et al.*, 2000; Giannakopoulos *et al.*, 2002; Jong Jeong *et al.*, 2003; Karagas *et al.*, 2005; McGrath *et al.*, 2006). These studies include various people from various geographic regions and of various ethnicities. Also, when stratified according to gender, no significant associations were found by McGrath *et al.* (2006). In addition, several of studies that stratified data according to smoking status did not find an association in

either non-smokers or smokers (Toruner *et al.*, 2001; Jong Jeong *et al.*, 2003; Karagas *et al.*, 2005).

Despite the findings in the aforementioned studies, data from other studies do suggest associations between the GSTT1 genotype and bladder cancer risk, however, these associations as discussed in Section 5.5.5 are likely a result of gene-gene interactions with the GSTM1-null genotype. Among a German population, Kempkes *et al.* (1996) found that non-smokers with the GSTT1-null genotype had a 3.84-fold increase in risk (95% CI = 1.21-12.23; $p = 0.023$). Although not statistically significant ($p = 0.09$), Salagovic *et al.* (1999) also found an almost 2-fold increase in bladder cancer risk among non-smokers having the GSTT1-null genotype. Similar results were also observed in more recent studies among North Indians and Swedes (Sanyal *et al.*, 2004; Srivastava *et al.*, 2004).

5.3.4 GSTP1

Whereas GSTP1is highly expressed in the urinary tract (Terrier *et al.*, 1990), associations between the GSTP1 genotype and bladder cancer risk are inconsistent and generally do not show an association. Several studies have shown no statistically significant associations between the GSTP1 genotype and bladder cancer risk. In studies conducted in Spain and Germany, no significant increases in bladder cancer were observed among the GSTP1 val105 variants (Steinhoff *et al.*, 2000; Garcia-Closas *et al.*, 2005). No association between the GSTP1 val105 variant and bladder cancer risk was also observed in studies that had considered smoking among Italians and Japanese (Katoh *et al.*, 1998; Hung *et al.*, 2004b). Hung *et al.* (2004b) also found

a lack of association between the GSTP1 genotypes and bladder cancer risk when considering occupational exposures to bladder carcinogens.

Despite the findings of the previously mentioned studies, other studies have suggested a significant association between the GSTP1 val105 variant genotype and bladder cancer risk. In a study on bladder cancer patients in the United Kingdom, researchers found a significant association between the GSTP1 val105 variant allele and bladder cancer was found (Harries *et al.*, 1997). Though the study was conducted on a small group of patients, the OR for the GSTP1 homozygous val105 variant genotype was 3.6 ($p = 0.006$). Given the patients included mostly smokers, these results are somewhat unexpected given the GSTP1 val105 variant is more efficient in conjugating diol epoxides. However, the variant may have lower catalytic activity toward particular compound(s) in tobacco smoke that are carcinogenic to the bladder (e.g., heterocyclic amines). Such findings could also be explained by exposures to an unidentified carcinogen metabolized by GSTP1 that is unique to the study population (Steinhoff *et al.*, 2000; Ma *et al.*, 2002). Finally, although less likely, it is possible that such results could be a result of a toxification rather than detoxification pathway. Similar results relating the increase in cancer risk with the GSTP1 val105 allele were also observed in Turkish and Indian populations with ORs of 1.75 (95% CI = 1.03-2.99; $p = 0.034$) and 7.12 (95% CI = 2.81-18.93; $p < 0.001$) (Toruner *et al.*, 2001; Srivastava *et al.*, 2005).

In addition to the associations found in the studies conducted in the UK and Turkey, the GSTP1 val105 variant was also shown to be non-significantly associated

with an increase in bladder cancer risk among Chinese workers exposed to benzidine (Ma *et al.*, 2002). This risk increase had an OR of 1.95 (95% CI = 0.70-5.46). Additionally, the GSTP1 variant was overrepresented in benzidine-exposed workers with modified exfoliated urothelial cells having an averaged Papanicolaou's cytological grading of greater than 2 (Ma *et al.*, 2003).

In contrast to the studies finding that the GSTP1 variant is associated with an increase in bladder cancer risk, a recent study by Cao *et al.* (Cao *et al.*, 2005) suggested that the GSTP1 ile105 wild-type allele was associated with an increase in bladder cancer with an adjusted OR of 7.5 (95% CI = 0.84-67.21) for the ile/ile genotype. Their data also suggested that the GSTP1 ile/ile105 genotype may modify the risks posed by smoking. It is important to point out that in these results the frequency of the GSTP1 val105 homozygotes was relatively small (i.e. 2 cases versus 11 controls) and that the frequency of the GSTP1 ile105 homozygotes was similar between cases and controls (53.1% versus 54.7%).

5.3.5 Gene-Gene Interactions

Gene-gene interactions are important considerations when evaluating potential associations between polymorphic enzymes and risk of bladder cancer. In fact, gene-gene interactions likely account for the associations observed between individual genotypes and bladder cancer risk.

With regards to GSTM1 and GSTT1, the combination of the null genotypes has been associated with significant increases in bladder cancer risk (Abdel-Rahman *et al.*, 1998; Salagovic *et al.*, 1998; Lee *et al.*, 2002; Hung *et al.*, 2004a; Srivastava *et*

al., 2004b). Several of these studies also found significant interactions between the combined null genotypes and bladder cancer risk (Figure 20). This would suggest that GSTM1 and GSTT1 play a complementary role in detoxifying bladder carcinogens.

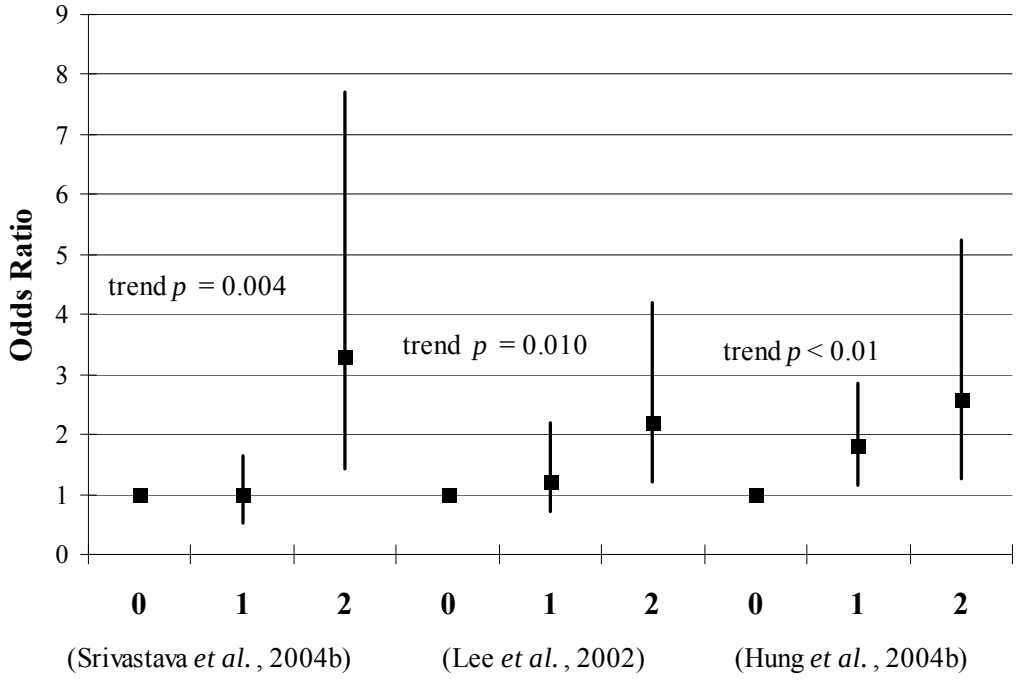

Figure 20. Relative Risk of Bladder Cancer for Combined GSTM1-null and GSTT1-null Genotypes. 0 = No Null Genotypes (reference); 1 = Either Gene Null; 2 = Both Null.

However, contrary to these findings, Katoh *et al.* (1998) found that the combination of the GSTM1-null and GSTT1-present genotypes was associated with an increase in cancer risk among a population of Japanese (OR =2.62; 95% CI = 1.36-5.05). No significant increase in risk was observed in the group containing both null genotypes. Such differences may be explained by exposure to an unknown bladder carcinogen, which is activated by GSTT1 (Katoh *et al.*, 1998). Similar, but non-significant

increases in risk were also observed by Brockmoller *et al.* (Brockmoller *et al.*, 1996a). In light of these findings other studies have found no association, suggesting that the interaction between GSTM1 and GSTT1 genotypes is weak and may rely on additional susceptibility factors (Giannakopoulos *et al.*, 2002; Kim *et al.*, 2000; Garcia-Closas *et al.*; 2005; Karagas *et al.*, 2005; and Steinhoff *et al.*, 2000).

When evaluating interaction between GSTP1 and other GSTs, results vary. A study by Hung *et al.* (2004) found no association between GSTP1 and other GST genotypes, whereas Toruner *et al.* (2001) found an association between the GSTM1-null and GSTP1 variant and an increase in cancer risk. Such differences may be explained by non-overlapping activity toward bladder carcinogens in tobacco smoke, and perhaps overlapping metabolic activity toward an unknown bladder carcinogen that was unique to the Turkish population studied by Toruner *et al.* (2001).

In addition to interactions among metabolic genes of the same family, gene-gene interactions and their associations with bladder cancer risk have been observed among genes of different families. In a study on German bladder cancer patients, researchers found that the GSTM1 non-null genotypes (1*0/1*1) in combination with slow acetylators with the genotype NAT2*5B/6A, increased bladder cancer risk significantly among men (Schnakenberg *et al.*, 2000). In women of the same genotypes, risks decreased significantly. The opposite was true for both groups among rapid acetylators. Such findings support the impact of gender on possible associations between the genotype and cancer risk, which may include sex-specific factors (e.g., hormones) and lifestyles that impact cancer development and disposition

(Schnakenberg *et al.*, 2000). However, interactions between GSTM1 and NAT genotypes were not observed by Giannakopoulos *et al.* (2002), Garcia-Closas *et al.* (2005), and Brockmoller *et al.* (1996a), but may be due in part to a lack of stratification according to gender. Given these results, gene-gene interactions as well as gene-gender interactions have the potential to be significant factors in associations between genetic polymorphisms and disease outcome in the bladder.

5.4 Parkinson's Disease

GSTs are expressed in the brain and have been shown to detoxify a wide range of substrates that may contribute to the development of PD, but the associations between the isozymes GSTM1, GSTT1, and GSTP1 and PD differ considerably. Data suggest that the role, if any, these enzymes partake in PD have little or no overlap. Such variability appears to stem from their expression levels in particular regions of the brain, substrate specificity, and the exact role the genotype may play in the development and/or progression of the disease.

5.4.1 GSTM1

The vast majority of studies support the notion that the GSTM1-null genotype is not associated with an increase risk of PD (Menegon *et al.*, 1998; Nicholl *et al.*, 1999; Ahmadi *et al.*, 2000; Rahbar *et al.*, 2000; Harada *et al.*, 2001; Kelada *et al.*, 2003; Deng *et al.*, 2004; Wahner *et al.*, 2007). However, a few studies do suggest that the genotype may be associated with PD risk and may be strictly dependent on environmental exposures, combination with other at-risk genotypes, or gender. In CYP2D6 poor metabolizers, the GSTM1-null genotype was associated with an

increase risk of PD (Santt *et al.*, 2004). In a population of men from the United Kingdom, the GSTM1-null genotype was associated with a significant increase in risk of PD, whereas no associations were found among women (Stroombergen and Waring, 1999). Researchers suggested that given the age group, such a difference may have been work-related. However, GSTM1 has not shown to be associated with PD risk among individuals exposed to high levels of pesticides (Menegon *et al.*, 1998).

Although the data overwhelming supports a minimal role for GSTM1 in the risk of PD, GSTM1 may still play a significant role in the onset and progression of the disease. In disease progression, the loss of the dopaminergic neurons results in enhanced metabolism of dopamine resulting in augmented formation of H_2O_2, which leads to the generation of hydroxyl radicals (Ebadi *et al.*, 1996). Given GSTM1 has some activity towards *o*-quinones of catecholamines, but much less compared to the GSTM2-2 isozyme (Baez *et al.*, 1997), its impaired ability to detoxify these *o*-quinones and other products of oxidative stress may further promote the disease progression and onset age. This is supported in a study by Ahmadi *et al.* (2000). Whereas no overall association was found between the genotype and PD risk, individuals having the GSTM1 gene had a significantly elevated median age for the onset of PD compared to those with the null genotype. The difference in the median age of onset between the two genotypes was 11 years. These findings were not observed in studies by Golbe *et al.* (2006) and Rahbar *et al.* (2000).

5.4.2 GSTT1

Overall, GSTT1 does not appear to play any significant role in the risk of Parkinson's disease. Except for one study, no associations between the null genotype and risk of idiopathic PD have been observed (Bandmann *et al.*, 1997; Menegon *et al.*, 1998; Ahmadi *et al.*, 2000; Kelada *et al.*, 2003; Deng *et al.*, 2004; Nishimura *et al.*, 2005; Wahner *et al.*, 2007). Potential explanations for these findings may include gene expression and substrate specificity. Assuming GSTT1 is not expressed in the tissue of concern and/or has no activity toward the substrates responsible in the development of PD, it is expected that an at-risk variant for the enzyme may not be a risk factor for disease originating from that tissue. This is supported in a study by Ahmadi *et al.* (2000) where they found an absence of GSTT1 gene expression in the substantia nigra from normal individuals. In comparison, the same study did find GSTM1 gene expression in substantia nigra tissue and found that the null genotype was associated with PD progression. In further support of this lack of association is a study conducted on specific activities of GSTs toward *o*-quinone products of catecholamines, including aminochrome, dopachrome, adrenochrome and noradrenochrome (Baez *et al.*, 1997). This study found that GSTT1 had no activity toward these intermediates of dopamine and adrenaline metabolism.

Despite the lack of association between the GSTT1 genotype and risk of PD, one study did find a significant association with idiopathic PD (Stroombergen and Waring, 1999). However, although not discussed by the authors, this could be a false

positive given a high percentage of the study individuals were also GSTM1-null (69% of the men had the GSTM1-null genotype). Of course this assumes that the GSTM1-null genotype is a risk factor and that a sufficient number of individuals carry both null genotypes.

5.4.3 GSTP1

GSTP1's potential association with PD is a bit more complex than the GSTM1 and GSTT1. GSTP1 is expressed in the brain as well as in the blood-brain barrier, and has shown little specific activity toward *o*-quinones of catecholamines (Carder *et al.*, 1990; Baez *et al.*, 1997). Additionally, when evaluating the at-risk genotypes association with PD independently, little or no association is observed (Kelada *et al.*, 2003; Wahner *et al.*, 2007). For these reasons, it may be expected that the genotype's association with PD risk may be dependent on exogenous factors, such as exposures to pesticides and other neurotoxic substances. This is supported in studies accounting for pesticide and smoking exposures (Menegon *et al.*, 1998; Deng *et al.*, 2004). In exposures to pesticides the val105 variant allele was significantly higher in idiopathic PD patients, suggesting that the coded enzyme had a decrease ability to detoxify the pesticides or an increase ability to convert the pesticide into a toxic metabolite (Menegon *et al.*, 1998). Similar to these results, Kelada *et al.* (2003) and Deng *et al.* (2004) found significant associations between GSTP1 genotypes and PD risk among smokers. Although smoking has shown to exert a protective effect against PD (Morens *et al.*, 1995; Checkoway *et al.*, 2002), variant genotypes were associated with a significant increase in risk of PD; however, the Kelada *et al.* (2003)

study found this association amongst carriers of the val105 carriers, whereas Deng *et al.* (2004) found the association amongst carriers of the 114Val variants. It was suggested that both of these variant genotypes may have a decreased ability to activate a particular compound of cigarette smoke that exerts a protective effect in the brain (Deng *et al.*, 2004).

The associations between GSTP1 polymorphisms and risk of PD may be modified by gender. This was observed by Kelada *et al.* (2003). When stratifying results according to gender, men having the heterozygous GSTP1 105 genotype were at a 2-fold (95% CI = 1.12-3.29) increased risk of PD, whereas no association was observed among women. Although the authors did not provide any explanation for their findings, gender may directly contribute to GSTP1 potential association with PD risk, which may involve differences in hormones or metabolic status. Another explanation, which was mentioned earlier, could be that the men were exposed to a greater amount of neurotoxins metabolized by GSTP1. This may too explain why this same study found that individuals having the heterozygous GSTP1 105 genotype over the age of 60 were at an increase risk of PD, while no association was found between individuals below 60 (Kelada *et al.*, 2003). Rather than the difference being a result of age or gender, these groups differ due to exposures to high levels of neurotoxins.

While age may not modify the association between GSTP1 genotype and risk of PD, GSTP1 genotype may impact the onset age of PD. In two available studies on onset age of familial PD, little or no associations are found among the GSTP1

variants alone and onset age of PD (Wilk *et al.*, 2006; Golbe *et al.*, 2007). When comparing the results of both studies, these potential associations appear to be very complex. The Wilk *et al.* (2006) study found no overall association between the val105 and val114 variants and onset age among familial cases of PD. However, when stratified according to pesticide exposure, the data suggests that individuals carrying the val114 variant allele may have an increase in onset age among individuals with occupational herbicide exposure. Interestingly, other SNPs of the GSTP1 gene showed significant trends for decreases in onset age among the herbicide exposure groups. When combined with the rs762803 SNP, which had the largest non-significant effect size (-9.58 years), the 114 wild-type allele was significantly associated with an earlier onset age of PD (-7.93 years; $p = 0.008$) among individuals exposed occupationally to herbicides (Wilk *et al.*, 2006). This same genotype also showed a borderline statistically significant delay in onset age for the no-exposure group (2.82 years $p = 0.048$). In comparison, all other combinations of these SNPs showed no statistically significant associations with onset age of PD in any of the exposure groups. Yet, a non-significant increase in onset age was observed in the occupational herbicide exposure group carrying the val114 variant combined with the variant SNP, rs762803.

In contrast to the findings by Wilk *et al.* (2006), another study suggested that the val114 variant is associated with an accelerated onset age of PD (Golbe *et al.*, 2007). This study was conducted on a group of Italians and Greeks with the α-synuclein A53T (PARK1) mutation, a causative factor in familial PD

(Polymeropoulos *et al.*, 1997). Researchers found a 9.7-year acceleration of the disease compared to the wild-type genotype with borderline significance (p = 0.0519). Additionally, although the group was very small (n = 3) this study found that the val105 homozygotes had a 15.2 year acceleration in onset age (p = 0.202) compared to carriers of at least one wild-type ile105 allele.

5.5 Liver Disease

5.5.1 GSTM1

GSTM1 is expressed in biliary epithelial cells, hepatic stellate cells and hepatocytes (Lakehal *et al.*, 1999; Whalen *et al.*, 1999). Given its distribution in the liver and broad range of substrates, the null genotype could be a susceptibility factor for a whole host of toxic insults to the liver. Despite this potential, data suggests that GSTM1's association with diseases of the liver is quite complex. Similar to mEH, associations tend to be dependent on multiple at-risk factors. This in part might be expected given GSTs share many of the same substrates, or are compensated for by other enzymatic systems. Also, the null genotype is not always the susceptible genotype to disease of the liver.

In hepatocellular carcinoma (HCC), GSTM1-null's association with this disease appears to be dependent on significant exposures to hepatotoxicants. In fact, when GSTM1 is evaluated alone or among groups exposed to low levels of potential hepatocarcinogens (i.e., aflatoxin), associations between the null genotype and risk of HCC are generally not observed. This lack of association is supported in several studies on aflatoxin-related hepatocellular cancer in Chinese and African populations

(Omer *et al.*, 2001; Sun *et al.*, 2001; Deng *et al.*, 2005; Kirk *et al.*, 2005). When exposures to aflatoxin were high, Sun *et al.* (2001) and Kirk *et al.* (2005), Omer *et al.* (2001), and Deng *et al.* (2005) found that GSTM1-null genotype was associated with a significant increase in HCC risk. No association was found between the GSTM1-null genotype and HCC risk among individuals with low levels of exposure to aflatoxin (Omer *et al.*, 2001; Sun *et al.*, 2001; Kirk *et al.*, 2005). Interestingly, a couple of these studies found that GSTM1-null without consideration to aflatoxin or other hepatotoxic substances or diseases was associated with a decrease in HCC risk (Sun *et al.*, 2001; Kirk *et al.*, 2005). This inverse association could be a result of GSTM1's conjugation and subsequent removal of chemopreventive chemicals, such as isothiocyanates (London *et al.*, 2000b; Sun *et al.*, 2001), which is only evident when exposures to toxicants is low.

Similar results have also been observed between the GSTM1-null genotype and risk of liver cancer in individuals who consume alcohol. Although alcohol has shown not be directly carcinogenic, it is a potential risk factor in that it is related to cirrhosis. Additionally, alcohol may also increase cancer risk through induction of microsomal enzymes that activate procarcinogens (Lieber *et al.*, 1986). Therefore, GSTM1s roles may include conjugation of activated procarcinogens and/or detoxification of reactive metabolites induced by oxidative stress. In studies on alcohol and HCC risk, the null genotype has been significantly associated with an increase in risk (Yu *et al.*, 1999; Covolo *et al.*, 2005). Furthermore, these studies found that these associations were found only in individuals who consumed large

amounts of alcohol. Yu *et al.* (1999) found that the GSTM1-null genotype was associated with a 2.6-fold (95% CI = 1.18-5.78) increase in HCC risk compared to non-drinkers of the same genotype. No significant increase in HCC risk was observed in drinkers compared to non-drinkers among individuals having the GSTM1-present genotype (Figure 21).

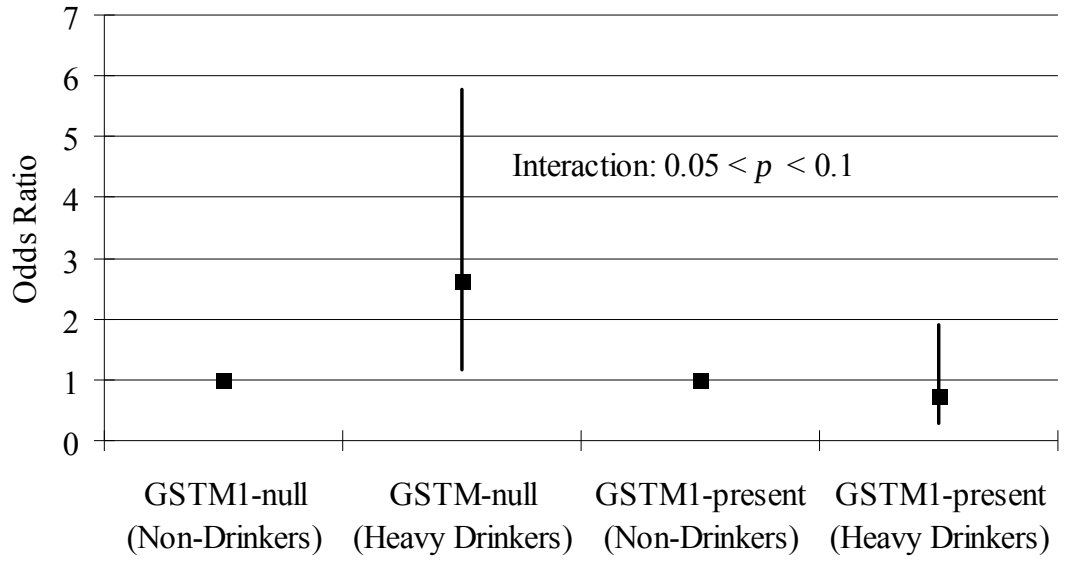

Figure 21. Relative Risk of HCC for GSTM1 Genotypes Among Non-Drinkers and Drinkers. Based on data from Yu *et al.* (1999).

These results were confirmed by Covolo *et al.* (2005) who found that carriers of GSTM1-null genotype combined with very high alcohol intake (> 100 grams/day) had a significant 8.5-fold increase in HCC risk (95% CI = 3.9-18.6) compared to low alcohol intake and the GSTM1-present genotype (Figure 22).

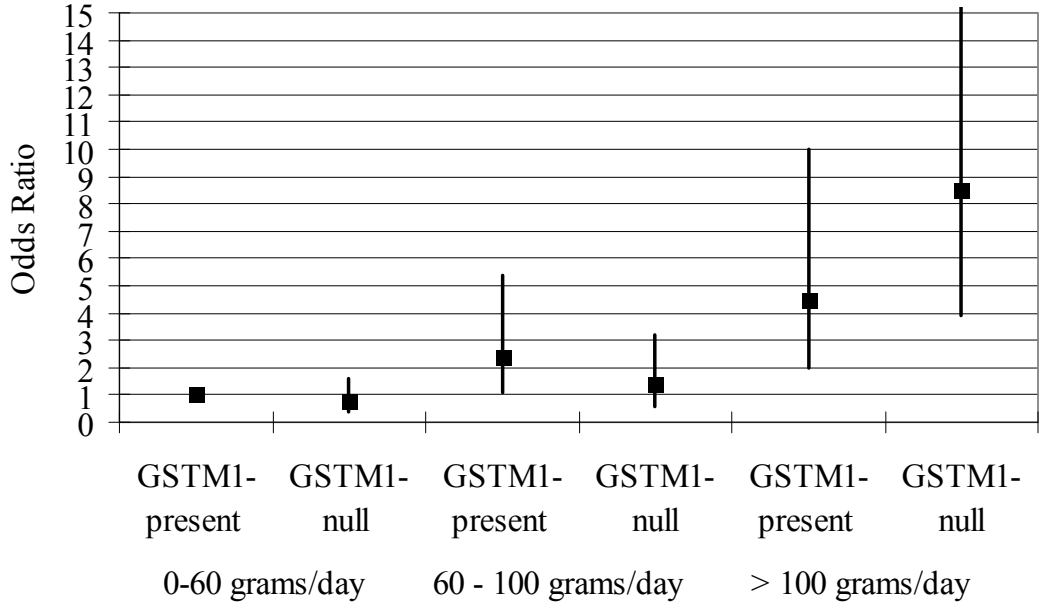

Figure 22. Relative Risk of HCC for GSTM1 Genotypes by Alcohol Consumption. Based on data from Covolo *et al.* (2005)

In comparison, the GSTM1-present genotype and very high alcohol intake imparted a significant increase in risk too, but lower with an OR of 4.5 (95% CI = 2.0-10.0). Associations between the genotypes and HCC risk were generally not observed between the genotypes and alcohol intake below 100 grams/day. Based on these findings, the GSTM1-null modifies the association between alcohol and HCC risk of HCC, but only at high doses with a significant interaction ($p = 0.02$) (Covolo *et al.*, 2005). This suggests that at lower doses other enzymes or metabolic pathways are able to provide sufficient cellular protection.

When considering other diseases of the liver, such as cirrhosis and fibrosis, data supports the notion that associations between these diseases and GSTM1 are also dependent on significant exposures to xenobiotics responsible for these diseases.

High alcohol intake and the risk of cirrhosis and fibrosis were found to be modified by the GSTM1-null genotype among Slavic and Finnish populations (Baranov *et al.*, 1996; Savolainen *et al.*, 1996). When evaluating moderate alcohol drinkers no association was found between the null genotype and risk of fibrosis (Savolainen *et al.*, 1996).

Along with GSTM1's potential role as a risk modifier for alcohol and aflatoxin-related liver diseases, GSTM1 modifies risks of liver toxicity in individuals exposed to high levels of drugs that cause injury to the liver. In a study on bone marrow transplantation patients undergoing conditioning therapy with busulfan and cyclophosphamide, the GSTM1-null genotype was found to be associated with an increase in hepatic venoocclusive disease (Srivastava *et al.*, 2004a). Similar results were also observed by Roy *et al.* (Roy *et al.*, 2001) in which the GSTM1-null genotype was associated with an increase risk of antituberculosis drug-induced hepatotoxicity in which GSTM1 may be involved in the conjugation of reactive metabolites of antituberculosis drug (e.g., isoniazid, rifampicin, pyrazinamide) metabolism.

Whereas the GSTM1-null genotype is not a risk factor of liver disease unless individuals are exposed to high levels of hepatotoxicants, antioxidants may modify these associations. In other words, low levels of antioxidants could lower the threshold for the null genotype being a susceptibility factor. In support of this notion are findings by Yu *et al.* (1999). While this study found that the GSTM1-null genotype was only associated with HCC risk among very high alcohol intake, the

study also observed that dietary antioxidant levels may modify this association. When stratified according to plasma carotenoid levels, the study found a significant increase in HCC risk among drinkers having the null genotype and low carotenoid levels. In drinkers with low β-carotene plasma levels, the ORs of HCC for null subjects and non-null subjects were 8.28 ($p < 0.01$) and 1.37, respectively. Similar results were also observed among smokers with low β-carotene plasma levels where the ORs for the null and non-null subjects were 3.54 ($p < 0.05$) and 1.34, respectively. No significant increases in HCC were observed in the high β-carotene groups (Yu *et al.*, 1999) (Figure 23). Similar results were also observed in other carotenoid groups including α-carotene and lycopene.

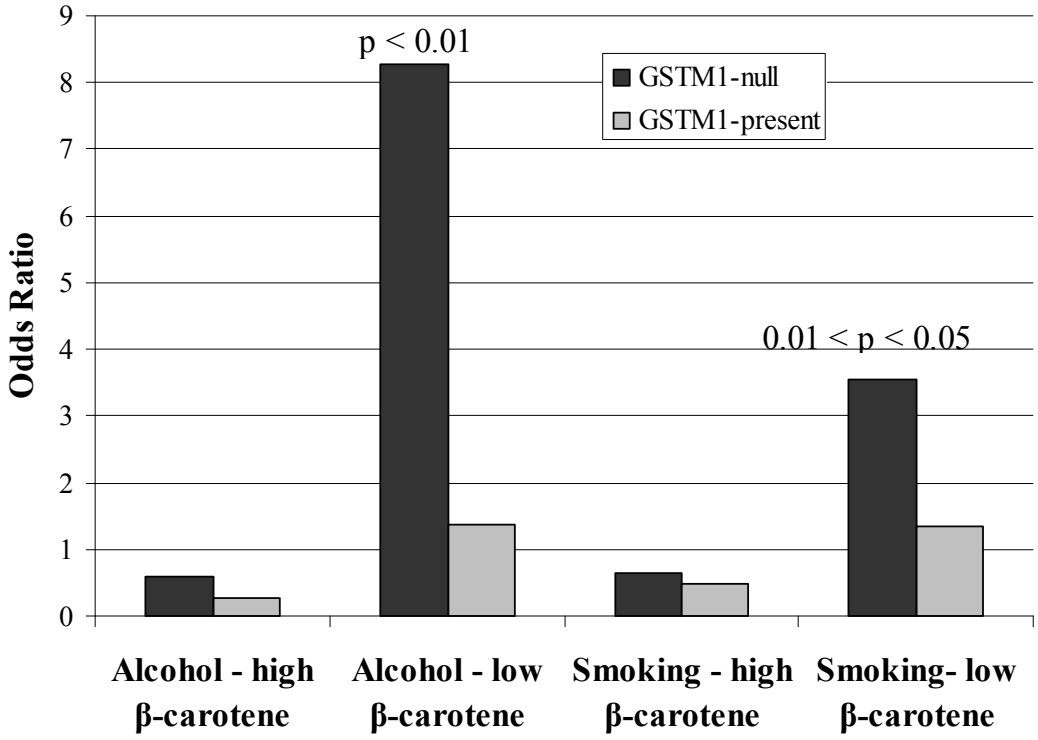

Figure 23. Relative Risk of HCC for GSTM1 genotypes at Various Levels of β-Carotene. Based on data from Yu *et al.* (1999).

Ethnicity may too play a factor in the presence or lack of association between the GSTM1-null genotype and risk of liver disease. This is suggested in a number of studies conducted on Caucasians. Note, a majority of the studies finding associations between the GSTM1 genotype and risk of liver disease were found in Asians and Africans with high nut consumption. In Spaniards of Caucasian decent, the GSTM1-null genotype was not associated with alcoholic liver disease or HCC, even after considering factors such Hepatitis B or C infection, tobacco use, and alcohol (Rodrigo *et al.*, 1999; Ladero *et al.*, 2005; Ladero *et al.*, 2006). Similar findings were also observed among Caucasians from France, Australia, United Kingdom, and Italy

(Groppi *et al.*, 1991; Frenzer *et al.*, 2002; Brind *et al.*, 2004; Gelatti *et al.*, 2005). Despite these findings, ethnicity may not explain this lack of association given studies on Finnish and Slavics have shown associations between the GSTM1-null genotype and liver disease. Additionally, no associations have been found among a population of Japanese HCC patients as well as in a heterogeneous population of Brazilian alcoholics with cirrhosis. Therefore, diet and inter and intra-ethnic factors may contribute to these differences.

5.5.2 GSTT1

GSTT1's role in liver disease susceptibility is uncertain. Overall, data suggests that GSTT1 is not significantly associated with liver diseases; however, data from available research yields inconsistent results. This may be due in part to the types of studies that focus on xenobiotics that may not be substrates for GSTT1 (e.g., AFB1-epoxide). Unlike GSTM1, GSTT1's potential substrates that induce liver toxicity may be limited to a smaller group or different set of xenobiotics; however, like GSTM1, GSTT1's association with disease risk is dependent on other genetic and environmental factors. In addition, data does support inverse associations (i.e., the non-null is a susceptibility factor).

Contrary to the links between GSTM1, alcohol, and risk of HCC, research supports that the GSTT1-null genotype alone is not significantly associated with an increase in HCC due to alcohol consumption (Yu *et al.*, 1999; Frenzer *et al.*, 2002; Munaka *et al.*, 2003; Covolo *et al.*, 2005; Ladero *et al.*, 2006). These results were observed in persons of different ethnicities and geographic locations including

Italians, Spaniards, Japanese, Chinese, and Australians. In addition, the amount of alcohol consumption did not modify this association (Covolo *et al.*, 2005). GSTT1's apparent limited role in alcohol-related liver disease is also supported in studies on the GSTT1 polymorphism and alcoholic liver disease (Frenzer *et al.*, 2002; Brind *et al.*, 2004; Burim *et al.*, 2004). Interestingly, the Brind *et al.* (2004) study found a statistically significant increase in alcoholic liver disease in one of the three subgroups of their study population. Each subgroup was from a different area in the United Kingdom. Such results suggest a false positive or perhaps it is due to a unique environmental exposure (Brind *et al.*, 2004).

Similar results are also observed in exposures to other potential causes of liver cancer, including exposures to aflatoxin and smoking. In African and Chinese HCC patients, Tiersma *et al.* (2001), Kirk *et al.* (2005) and Long *et al.* (2006) did not find a significant association between the null genotype and risk of HCC. The null genotype was also found not to modify the association between smoking and liver cancer among Spaniards (Ladero *et al.*, 2006).

While these findings suggest the GSTT1 genotype alone is not significantly associated with liver cancer and other liver diseases, the GSTT1 genotype may be associated with increases in liver cancer in conjunction with other at risk genotypes, chronic disease, and other environmental and dietary factors. First, the amount of exposure to potential hepatotoxicants may play a significant role in the GSTT1-null genotype being a risk factor for diseases and injury to the liver. In workers exposed to dimethylformamide (DMF), Luo *et al.* (Luo *et al.*, 2005) found a significant

increase in abnormal liver function in workers exposed to high levels of DMF having the GSTT1-null genotype.

A similar high dose requirement may also be required for hepatocarcinogens, such as aflatoxin. In a study conducted on Chinese HCC patients residing in an area highly contaminated with aflatoxin, researchers found a significant association between the null genotype and increased risk of cancer (Deng *et al.*, 2005). However, a subsequent study from this same region of China found no association between the GSTT1-null genotype and increase in HCC (Long *et al.*, 2006). Sun *et al.* (2001) also found no significant interaction between aflatoxin exposure and GSTT1-null in HCC risk. Therefore, high doses to potential hepatotoxicants may not be the only factor that results in the GSTT1-null genotype being a susceptibility factor for HCC. Perhaps GSTT1 is not a significantly involved in the detoxification of aflatoxin, which may explain these findings.

Similar to GSTM1, dietary intake of antioxidants may modify the association between the GSTT1-null genotype and risk of HCC. This is supported in a study by Yu *et al.* (1999). Smokers with low β-carotene plasma levels having the GSTT1-null genotype had a statistically significant increase in HCC risk (OR = 3.06; $p < 0.05$), whereas no significant increase was observed in smokers with the GSTT1-present genotype. However, unlike the observations found in alcohol drinking among the GSTM1 genotypes, the GSTT1-null genotype did not affect the risk of HCC among drinkers with low β-carotene levels. The ORs for the GSTT1-null and GSTT1-

present genotypes with low β-carotene levels among drinkers was 2.91 ($0.05 < p < 0.1$) and 4.22 ($0.01 < p < 0.05$), respectively (Yu *et al.*, 1999) (Figure 24).

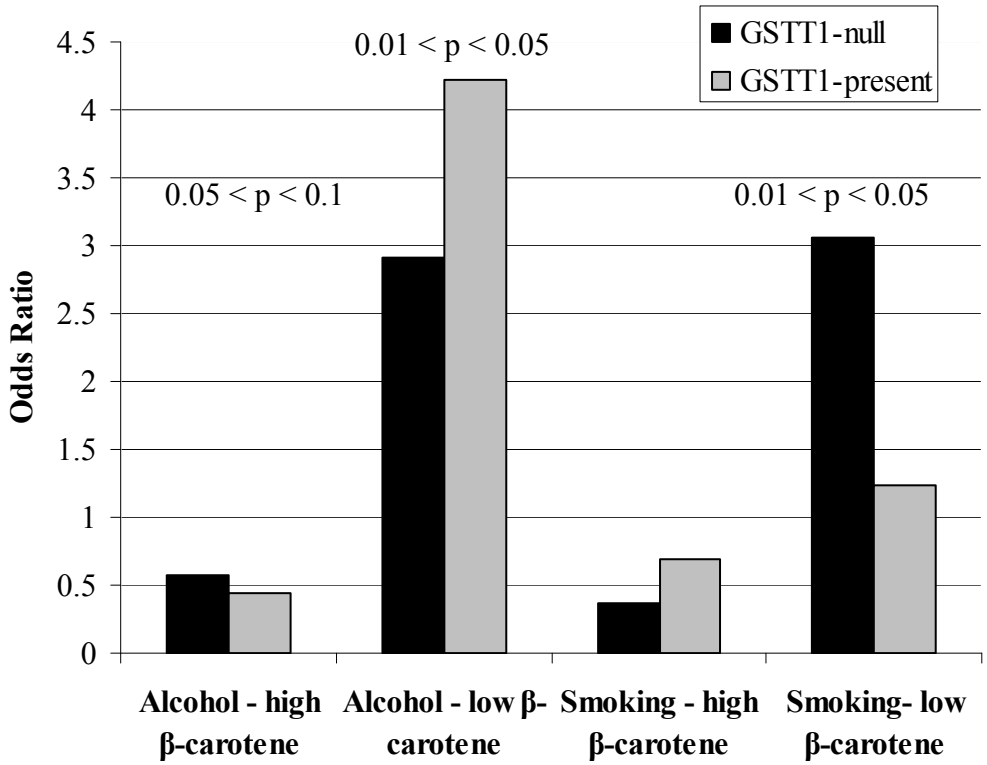

Figure 24. Relative Risk of HCC for GSTT1 genotypes at Various Levels of β-Carotene. Based on data from Yu *et al.* (1999).

The GSTT1 genotype's association with liver cancer may also be dependent on multiple risk factors, including inflammatory disease (e.g. HBV) and exposures to high levels of carcinogens. Although studies are very limited, the GSTT1-null genotype has been shown to be associated with an increase in HCC among hepatitis B carriers who were exposed to high levels of aflatoxin (Chen *et al.*, 1996; Sun *et al.*, 2001). In the Sun *et al.* (2001) study, researchers found that the increase in risk was only observed when all factors were considered (OR = 3.7; 95% CI = 1.5-9.3). When

considering HBV and the GSTT1 genotype alone, they found that the GSTT1-null genotype was significantly lower in HCC cases. Such an association is somewhat unexpected and has not been observed in other studies that found no significant association between the GSTT1 genotype and risk of liver disease among individuals with hepatitis B or C infection (Ladero *et al.*, 2006; Mohammadzadeh Ghobadloo *et al.*, 2006).

Along with dose, diet, and disease status being important factors in whether the GSTT1 genotype is associated with hepatotoxicity, the biotranformation pathway used to detoxify or activate a particular xenobiotic may be an important factor. When studying potential associations between GSTT1 and polyvinyl chloride (PVC) workers exposed to high and low levels of vinyl chloride in China, Huang *et al.* (1997) was able to show that the metabolic pathway may determine the type of association the GSTT1 genotype has with toxic responses of the liver. Vinyl chloride's biotransformation has shown to be dose dependent and at high concentrations vinyl chloride is thought to be oxidized by cytochrome P450 2E1 into an epoxide intermediate, which can be spontaneously rearranged into an aldehyde (2-chloroacetylaldehyde) (Guengerich *et al.*, 1991; Easter and Von Burg, 1994). At lower doses, vinyl chloride is thought to be oxidized by alcohol dehydrogenase to form chloroethanol, 2-chloroacetylaldehyde, and chloroacetic acid (Easter and Von Burg, 1994). In the Huang *et al.* (Huang *et al.*, 1997) study, researchers found that the positive genotype was significantly higher in workers with abnormal liver function (OR = 3.8; 95% CI = 1.2-14.5) whom were exposed to low levels of vinyl

chloride. At high doses, the positive genotype was significantly lower in workers with abnormal liver function (OR = 0.3; 95% CI = 0.1-0.9). These findings suggest that at low doses both GSTT1 and alcohol dehydrogenase metabolized vinyl chloride, whereby GSTT1 likely activated the vinyl chloride, which is consistent with GSTT1's ability to activate some classes of halomethanes (Thier *et al.*, 1993). At higher doses, CYP2E1 was primarily involved in oxidation of vinyl chloride, and null individuals were unable to conjugate the epoxide intermediates. Similar associations between the GSTT1-null genotype being a risk factor for vinyl chloride-related liver lesions were observed by Zhu *et al.* (2005).

The findings by Huang *et al.* (1997) may also help to explain why the GSTT1-present genotype was associated with increases in HCC risk in a couple studies in China (Bian *et al.*, 2000; Sun *et al.*, 2001). These studies found that the risk of HCC among the non-null genotype increased significantly. The Bian *et al.* (2000) study found an increase in risk with an OR of 4.13 (95% CI = 1.64-10.70) for carriers of the GSTT1-present genotype. Likewise, Sun *et al.* (2001) found that the null genotype was associated with a decrease in risk with an OR of 0.5 (95% CI = 0.2-0.9). Of considerable interest, which was mentioned previously, was that the GSTT1-null genotype in the Sun *et al.* (2001) study was associated with an increase in HCC risk with high exposures to aflatoxin and carriers of HbsAg. Here GSTT1 may be involved in bioactivating an unidentified environmental procarcinogen(s), but when individuals are exposed to high doses of carcinogens that the enzyme is involved in

detoxifying, a lack of this enzyme may be a susceptibility factor given other enzymatic systems may be saturated.

5.5.3 GSTP1

Research on associations between GSTP1 and hepatotoxicity is very limited. Presently, there is only one study that investigated associations between HCC and the GSTP1 genotype. In this study, Munaka *et al.* (2003) found no association between the GSTP1 genotype and HCC. Despite the lack of data on GSTP1 and HCC risk, more data is available on GSTP1's potential associations with other diseases of the liver. Though data is limited, such associations are shown to be dependent on significant exposures to hepatotoxicants, such as alcohol, and disease status.

Existing studies support that the GSTP1 val105/val105 genotype is associated with cirrhosis. Based on available research, data suggests that GSTP1's role may include a decreased ability to deactivate reactive metabolites of lipid peroxidation resulting from oxidative stress, whether it is due to endogenous or exogenous factors. The variant genotype was associated with a significant increase in cirrhosis among patients with hemochromatosis (Stickel *et al.*, 2005). The GSTP1 val105 variant has also shown to be associated with a significant increase in risk of cirrhosis among individuals infected with the hepatitis B virus (Mohammadzadeh Ghobadloo *et al.*, 2006). Additional data also supports that the val105 variant is associated with an increase risk of cryptogenic cirrhosis (Ghobadloo *et al.*, 2004). Nonalcoholic steatohepatitis is thought to be a major cause of cryptogenic cirrhosis, and steatohepatitis results in increased oxidative stress (Pessayre *et al.*, 2002; Portincasa

et al., 2006). When considering exogenous factors, similar results were also observed in alcoholics with cirrhosis (Burim *et al.*, 2004).

Contrary to these findings, one study found that the GSTP1 ile105/ile105 genotype (wild-type) was a risk factor for hepatotoxicity. In a study on liver disease in pediatric patients with cystic fibrosis, researchers found that the wild-type genotype was significantly higher in patients with liver disease, with an adjusted OR of 4.54 (95% CI 1.57-13.17) (Henrion-Caude *et al.*, 2002). The authors postulated that the GSTP1 wild-type had a decreased ability to detoxify lipid peroxidation products resulting from cystic fibrosis-related bile duct injury. While these findings contradict other studies, such findings suggest that these associations may be much more complex and be modified by many other factors. Although not addressed in the Henrion-Caude *et al.* (2002) study, these conflicting results may be due to another mechanism of cystic fibrosis-related liver injury. Enzyme activity, drug absorption and drug clearance have been shown to be affected in cystic fibrosis patients.

While the previous studies suggest that the GSTP1 genotype may be associated with diseases of the liver, Brind *et al.* (2004) found no association with ALD. However, this study excluded biliary cirrhosis where GSTP1 is highly expressed. Therefore, these results do not exclude the GSTP1 genotype from being a risk factor for diseases of the liver. Instead they support that any relation between genotype and liver disease risk is dependent where liver injury occurs and whether enzyme expression is significant in those areas.

5.5.4 GSTs and Gene-Gene Interactions in Liver Disease

As mentioned previously, the liver is a rich source of enzymatic activity, which is essential for its role as a biological "filter". Having this large array of biotransformation enzymes may in part explain many of the inconsistencies in associations between the GST, mEH, and NQO1 genotypes and diseases of the liver. Many of these enzymes have overlapping substrates, and therefore lack or decreased activity in one enzyme could be compensated for by another. Furthermore, at-risk genotypes could be compensated for by other mechanisms, such as DNA repair enzymes, etc. For these reasons it is especially important to consider gene-gene interactions when evaluating risk factors for disease. Indeed, evaluating gene-gene interactions in the context of liver disease susceptibility provides greater insight on whether a particular genotype is truly a risk factor for liver disease. Additionally, other factors, including the diet, environment, and ethnicity may play a significant role in modifying gene-gene interactions that contribute to liver disease.

The combination of the GSTT1-null and GSTM1-null genotypes has shown to significantly increase the risk of alcoholic liver disease and hepatocellular carcinoma, suggesting a shared role in detoxifying metabolites and products of aflatoxin and ethanol metabolism. Deng *et al.* (2005) reported that the combination of the null genotypes was significantly associated with a two-fold increase in HCC, resulting from high exposures to aflatoxin. Of particular interest is that the researchers did not find an association between the combination of the GSTM1-null and GSTT1-non-null or GSTM1-non-null and GSTT1-null genotypes with an increase in HCC. As

discussed in previous sections, the Deng *et al.* (2005) study reported that both genotypes were individually associated with HCC. However, these observations were made without consideration of the other GST genotypes; therefore, the combination of at-risk genotypes contributed to the observations made on the individual genotypes. This may have been a factor in other studies showing associations between GSTM1 and GSTT1 and aflatoxin-related HCC.

The combination of GSTM1-null and GSTT1-null genotypes have also been shown to be significantly associated with an increase in alcoholic liver disease, suggesting a shared role in detoxification of products resulting from oxidative stress. In a study on Spaniards, the combination of the null genotypes was significantly associated with an increase risk, with an OR of 4.3 ($p = 0.0003$) (Ladero *et al.*, 2005). Alone, GSTM1-null was not associated with alcoholic liver disease and GSTT1 null was associated with a slight, but statistically significant increase in risk (OR = 1.67; 95% CI = 1.03-2.71).

Similar results have also been observed in other toxic effects of the liver. Simon *et al.* (Simon *et al.*, 2000) found that combination of the GSTM1-null and GSTT1-null genotypes was associated with an increase in susceptibility to tacrine hepatotoxicity. Alone, neither GSTM1 nor GSTT1 genotypes were associated as a susceptibility factor. Similar results were also observed in a study on troglitazone-associated hepatotoxicity among type 2 diabetics (Watanabe *et al.*, 2003). These results further support that one at-risk genotype may not impart any increase in

disease risk when the enzyme it encodes shares a common substrate with another fully functional enzyme.

Gene-gene interactions where the transcripts (i.e., the enzymes) do not share common substrates could contribute to an increase or decrease in liver disease. This is supported by a study conducted by Tiersma *et al.* (2001) on GSTM1 and GSTT1 and mEH exon 3 and exon 4 polymorphisms in aflatoxin-associated HCC. Although this study confirmed the lack of association between aflatoxin exposure and HCC for the mEH and GSTT1 genotypes, they found that the combination of the GSTM1-present and mEH low-activity genotypes was associated with an increased risk of HCC (OR = 5.7; 95% CI = 1.2-28.2). This was unexpected given both GSTM1 and mEH can both detoxify the AFB1-epoxide. Similar results were also observed in the combination of the GSTT1-present and mEH low-activity genotypes (OR = 22.2; 95% CI = 2.4-205.8). Although the authors could not rule out these findings to chance, given the small sample groups, these findings suggest that the combination of variant forms of mEH, GSTM1, and GSTT1 could act to increase HCC risk through different mechanisms. The Tiersma *et al.* study (2001) data suggests that the mEH variant contributed to an increase in HCC risk, as a result of a lack of activity in a metabolic pathway other than AFB1 detoxification (Tiemersma *et al.*, 2001). GSTT1 and GSTTM1 could be involved in the activation of an unknown procarcinogen that the study group was exposed to, or the GSTs may be involved in the removal of chemopreventive compounds such as isothiocynates (Yu *et al.*, 1998).

In addition to the direct and indirect interactions between the biotransformation enzymes that contribute to increases in liver disease, at-risk GST genotypes may indirectly interact with at-risk genotypes of the enzymes not involved in detoxification pathways, such as DNA-repair enzymes. This was confirmed by Kirk *et al.* (2005) in a study on HCC in an African population with aflatoxin exposure and HBV endemicity. As discussed earlier, the GSTM1-null was associated with significant increase of risk of HCC, whereas the GSTT1 and mEH (exon 3) variants were not associated with significant increases in risk. However, the data did show a slight non-significant increase in HCC risk. When the researchers combined these genotypes with the variant DNA-repair enzyme XRCC1 genotype (heterozygote), which itself imparted a statistically significant increase in HCC risk (Adjusted OR (AOR) = 2.66 95% CI = 1.17-6.08), the risks increased dramatically. AORs for the GSTM1 and XRCC1 variants and the mEH and XRCC1 variants were 9.14 (95% CI = 2.20-38.0) and 5.89 (95% CI = 1.36-25.6), respectively. A combination of all three at-risk genotypes yielded an OR of 14.7 (95% CI = 1.27-169) (Figure 25).

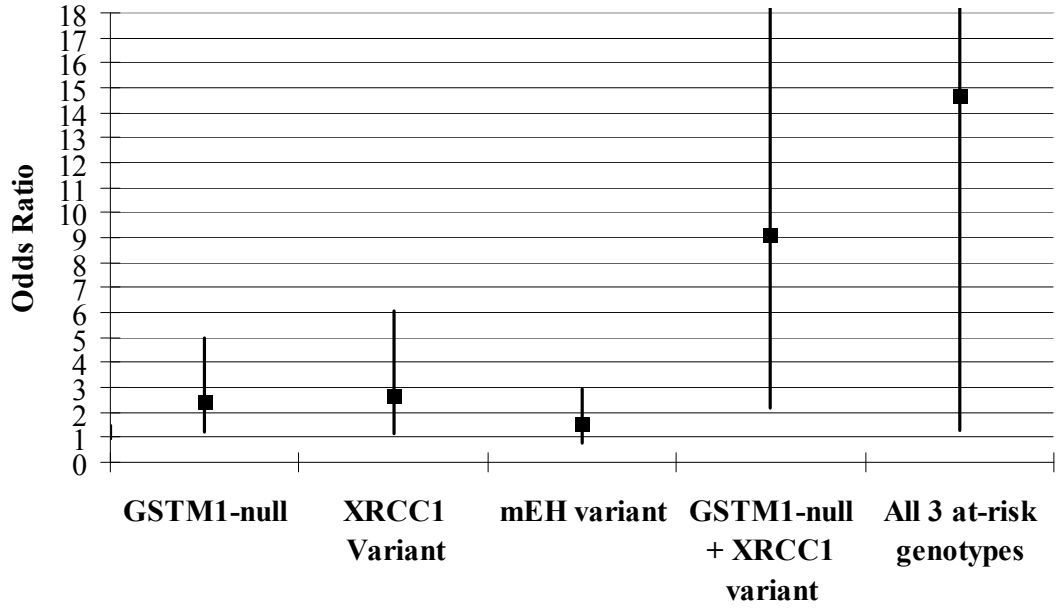

Figure 25. HCC risk for Combinations of mEH, GSTM1, and XRCC1 Polymorphisms. Based on data from Kirk *et al.* (2005).

Similar results were also observed by Long *et al.* (2006) among Chinese exposed to high levels of aflatoxin, where they found a statistically significant increase in HCC risk among the combination of all three at-risk genotypes (OR = 10.83; 95% CI = 5.44-21.59). These results suggest that biotransformation and repair enzymes can interact via a common pathway that leads to hepatocarcinogenesis.

Similar results showing the impact of DNA-repair enzymes on the association between biotransformation enzyme genotype and HCC risk have also been found among HBV carriers. A study by Yu *et al.* (2003) found that only after combining the GSTT1 null with the XRCC1 variant did risks of HCC increase. GSTT1-null genotype alone was not associated with an increase in HCC risk, nor did the XRCC1 variant in combination with the GSTT1 present show an increase in risk. The

combined GSTM1-null and XRCC variant, while non-significant, suggested that the null genotype decreased risks of HCC. When all three genotypes were combined, the greatest risk was found in the GSTT1-null, GSTM1 present, and XRCC1 variant. An increase in risk was also observed in the combination of the GSTT1-null, GSTM1-null, and XRCC1 variant genotypes. When considering the GSTT1 present, GSTM1-null or present, and XRCC1 variant, no increase in risks of HCC were observed. These results further support an interactive role of the biotransformation and DNA-repair enzymes genotypes. In this case, the GSTT1-null genotype is unable to protect cells from by-products of lipid peroxidation and oxidative stress resulting from HBV infection, which cannot be kept in check by a XRCC1 variant with a lower DNA-repair capacity (Yu *et al.*, 2003). The GSTM1 enzyme may be involved in removal of dietary antioxidants. As a result, there is a significant increase in risk of HCC.

5.6 Summary

As shown, susceptible GST genotypes are associated with increases in diseases of the lung, liver, and bladder. The associations with PD remain uncertain. Yet, associations between susceptible GST genotypes with disease of the lung, bladder and liver tend not to be observed without consideration of multiple susceptibility factors, including low dietary intake of antioxidants, disease, and other susceptibility genotypes. Of course, given that most of the diseases covered in this thesis are environmentally-related (e.g., tobacco smoking and lung cancer), exposures to high levels of xenobiotics are important as well.

5.6.1 GSTM1

When evaluating isozymes separately, currently available data suggests that the GSTM1 genotype is more closely tied to diseases of the lung, liver, and bladder compared to the other genotypes. In each of these organs, environmental and gene-gene interactions are determining factors in whether the GSTM1-null genotype is a susceptibility factor. No significant observations were made with PD. A summary of associations between GSTM1 and the diseases studied in this thesis are provided in Table 4.

Table 4. Summary of Associations Between GSTM1 Genotype and Disease

	GSTM1-null	GSTM1-present
Lung Cancer	↑ Slight, independent of smoking. Risk increase is attenuated with high isothiocyanate intake	Could be a slight risk factor due to removal of dietary isothiocyanates
Lung Disease	↑ Asthma especially in combination with GSTT1-null	-
Bladder Cancer	↑ Smokers especially in combination with GSTT1-null	-
PD	Unknown	-
Liver Cancer	↑ In combination with multiple susceptibility factors including hepatitis, mEH low activity genotype, and variant XRCC1 genotype	Could be a slight risk factor due to removal of dietary isothiocyanates
Liver Disease	↑ In combination with multiple susceptibility factors including hepatitis, high alcohol intake. Risk Increase may be attenuated with high antioxidant intake	-

↑ = Increase in Risk, ↓ = Decrease in Risk, "-" = Reference Group

In the lung, the GSTM1-null genotype is weakly associated with an increase in lung cancer risk and is more strongly associated with asthma risk. In regards to lung cancer, the GSTM1-null genotype's association with this disease is independent of smoking status. Additionally, supporting a weak association, high dietary intake of isothiocyanates attenuates any increase in risk. Interestingly, the GSTM1-present genotype may confer an increase risk among high dietary isothiocyanate intake compared to GSTM1-null genotypes as a result of conjugation of these compounds. Despite this overall weak association, gene-gene interactions modify these associations where individuals carrying susceptible CYP1A1 and NAT genotypes have a significant increase in risk of lung cancer.

As for asthma, individuals having the null-genotype tend to have greater increases in risk of this disease. However, these associations are slightly uncertain. *In utero* exposure cannot be ruled out as a contributor to increased asthma risk among GSTM1-null individuals. Additionally, associations between the GSTM1-null genotype and risk of asthma may be a result of individuals also having the GSTT1-null genotype as found by Ivaschenko *et al.* (2002).

Unlike the lung, the GSTM1-null genotype is more strongly associated with an increase in bladder cancer risk and is more closely associated with smoking. Significant increases in bladder cancer risk have also been observed in occupational exposure to bladder carcinogens, but some studies have found no associations. Gender may also modify the association between GSTM1-null and risk of cancer with female smokers carrying the null genotype being at a greater risk compared to male

smokers. When considering gene-gene interactions, much greater increases in cancer risk have observed between the GSTM1-null genotype and risk of bladder cancer among individuals with GSTT1-null genotype. Similar increases in bladder cancer risk have also been found with a combination the NAT genotype. However, gender determined which NAT and GSTM1 genotypes were risk factors for men and women.

In liver, where GSTM1 is highly expressed, the GSTM1-null genotype tended to be only associated with liver cancer and cirrhosis when combined with other at risk factors including high alcohol intake, HBV, HCV, and low dietary intake of antioxidants. Also, significant increases in liver disease risk were generally only observed when multiple susceptibility factors were included. Gene-gene interactions also substantially increased the risk of liver cancer among carriers of the GSTM1-null genotypes. Such findings were observed with concurrent lack of the GSTT1 gene. Furthermore, liver cancer risks increase dramatically among GSTM1-null genotype individuals carrying the susceptible genotype of the DNA-repair enzyme XRCC1.

5.6.2 GSTT1

Likely due to the diseases and organs studied in this thesis, the GSTT1 genotype tends not to be associated with increases in disease of the lung, bladder, and liver (Table 5). However, in combination with the GSTM1-null genotype it has been associated with an increase in risk of asthma, bladder cancer, and liver cancer, where they likely share common substrates, such as phospholipid hydroperoxide, a product of oxidative stress. In lung cancer, the combination of these genotypes and risk

increase has generally not been observed, which is expected given lung carcinogens such as BPDE are not GSTT1 substrates.

Whereas the GSTT1 genotype has been weakly associated with increase risk of disease, this genotype has shown how the level of exposure can determine whether a genotype is a risk factor or protective factor. GSTT1 null was a risk factor for liver lesions for workers exposed to high levels of vinyl chloride in that it was unable to conjugate epoxide intermediates of vinyl chloride resulting from CYP2E1 oxidation. At low exposure levels, the GST1 non-null was a risk factor in that it bioactivated vinyl chloride, which is consistent with its ability to bioactive some low-molecular-weight halogenated compounds.

Table 5. Summary of Associations Between GSTT1 Genotype and Disease

	GSTT1null	**GSTT1-present**
Lung Cancer	Overall Lack of Association	-
Lung Disease	↑ Asthma in combination with the GSTM1-null	-
Bladder Cancer	↑ In combination with GSTT1-null	-
PD	Unknown	-
Liver Cancer	↑ In combination with multiple susceptibility factors	↑ Bioactivation of halogenated alkanes, which is dependent on dose.
Liver Disease	↑ In combination with multiple susceptibility factors	-

↑ = Increase in Risk, ↓ = Decrease in Risk, "-" = Reference Group

5.6.3 GSTP1

Contrary to its expression levels in some tissues, including the lung and bladder, the susceptible GSTP1 genotype has shown not to be a significant risk factor (Table 6). Perhaps this is due a lack of studies on the enzyme compared to GSTM1 and GSTT1, or that the polymorphisms evaluated does not significantly impact the overall ability for the enzyme to detoxify reactive compounds. Differential affinity for substrates may affect any association between the GSTP1 genotype and disease too, especially in cancer where multiple compounds may play a role in the initiation and progression of the disease. For example, the GSTP1 val105 variant is less active toward a cancer initiator and more active toward products of oxidative stress.

While the GSTP1 has not shown to be significantly associated with many of the diseases studied in this thesis, it has been associated with a few. In combination with the GSTM1-null and low activity mEH genotypes, GSTP1 was associated with an increase risk of COPD. When exposed to high levels of hepatotoxicants, the GSTP1variant and wild-types have been associated cirrhosis and cystic fibrosis-related hepatoxicity, respectively, which provides a clear example of differential susceptibility for the GSTP1 genotypes. In PD, the GSTP1 105 variant allele has shown to be a potential risk factor, in particular among individuals exposed to pesticides. Of greater interest, is that studies on GSTP1 and PD have investigated the impact of other SNPs within the GSTP1 gene and their impact on PD. Although data is very limited, these other SNPs and the GSTP1 variant 105 genotype may be associated with accelerations of on-set age of PD. On a more global scale, the

combination of these SNPs may be better indicators of the overall activity of the GSTP1 enzyme and should be subject to further research.

Table 6. Summary of Associations Between GSTP1 Genotype and Disease

	GSTP1 val105 (Variant)	**GSTP1 ile105 (Wild-Type)**
Lung Cancer	Overall Lack of Association	Overall Lack of Association
Lung Disease	-	↑ Asthma in combination with the GSTM1-null
Bladder Cancer	↑ In combination with GSTT1-null	-
PD	↑ With exposures to pesticides	-
Liver Cancer	Uncertain	-
Liver Disease	↑ In combination with multiple susceptibility factors	↑ Certain diseases such as cystic fibrosis-related bile duct injury

↑ = Increase in Risk, ↓ = Decrease in Risk, "-" = Reference Group

Chapter 6: Nrf2

6.1 Introduction

Of all the polymorphisms evaluated in this thesis, polymorphisms in the Nrf2 that elicit decreased function could have the most profound effects overall given it role. However, the impacts of polymorphisms in the gene encoding Nrf2 are not currently known. Only one study was found in the published literature evaluating Nrf2 polymorphisms in a small number of patients with COPD or systemic lupus erythematosus (Yamamoto *et al.*, 2004). Despite this lack of information, this chapter will provide a brief summary of the potential impacts that a dysfunctional Nrf2 could elicit. However, it should be pointed out that the research that has been conducted has generally been performed on knockout mice. Therefore, the findings in these studies represent a "worst-case" scenario, and merely suggest the potential impacts a deficient Nrf2 protein could have in humans.

6.2 Lung Disease

Several animal studies have shown that Nrf2, which is highly expressed in the lungs, protects the lung from diseases and illnesses including COPD, acute respiratory distress syndrome, hyperoxic injury and pulmonary fibrosis. Mice deficient in Nrf2 were much less tolerant to the pulmonary toxicity of the antioxidant butylated hydroxytoluene (Chan and Kan, 1999). Knockout mice had a higher mortality rate brought on by asphyxia due to lung failure caused by loss of alveolar architecture with pulmonary infiltration and hemorrhage. Upon further examination

of these Nrf2 -/- mice, a decreased expression of several detoxification enzymes including NQO1 was observed.

When Nrf2 knockout mice are exposed to common pollutants, such as cigarette smoke and diesel exhaust, lung injury occurs. Nrf2 knockout mice exposed to cigarette smoke for 6 months had more pronounced bronchoalveolar inflammation and an increased number of apoptotic alveolar septal cells (Rangasamy *et al.*, 2004). Aoki *et al.* (2001) also found that mice exposed to diesel exhaust had severe hyperplasia and increased DNA adducts, including the accumulation of the oxidative DNA adduct 8-hydroxydeoxyguanosine in the bronchial epidermis. Based on these findings, researchers concluded that Nrf2 knockout mice were unable to protect cells from accumulation of ROS (resulting from the nitrogen dioxide component of diesel exhaust) and PAHs, which are responsible for the DNA adduct 8-hydroxydeoxygruanosine (Aoki *et al.*, 2001).

Additional studies by Cho *et al.* (2002 and 2004) have found the Nrf2's role in pulmonary fibrosis and hyperoxic lung injury. When mice were exposed to the anti-neoplastic agent bleomycin, lung injury and fibrosis markers were significantly attenuated in mice with wild-type Nrf2 (Cho *et al.*, 2004). This study also found the up-regulation of glutathione and a significant increase in GST alpha in Nrf2 wild-type mice. Nrf2 was also found to be a protection factor against hyperoxic lung injury where hyperoxia-induced mRNA levels of NQO1, GST, and other enzymes decreased significantly in Nrf2-knockout mice (Cho *et al.*, 2002). In addition, the basal NQO1 activity was 50% greater in the lungs of wild-type mice compared to the knockouts.

Total GST activity in the wild-type mice exposed to air or hyperoxia was also significantly higher compared to the knockout mice (Cho *et al.*, 2002).

6.3 Bladder

Nrf2 likely plays a significant role in bladder too. Nrf2-deficient mice exposed to urinary bladder-specific carcinogen *N*-nitrosobutyl (4-hydroxybutyl) amine (BBN) had a significant higher incidence of urinary bladder carcinoma than wild-type mice (Iida *et al.*, 2004). Bladder cancer was found in 24.0% of the wild-type mice and 38.5% in Nrf2 knockout mice. When mice were exposed to the phase 2 enzyme inducer oltipraz, the incidence of urinary bladder carcinoma by BBN in wild-type mice decreased, but had little effect in Nrf2-/- mice.

6.4 Parkinson's Disease

Though studies are lacking regarding Nrf2 polymorphisms and risk of PD, Nrf2's control of antioxidant and biotransformation pathways may play a pivotal role in cellular protection and defense against PD. Such a role is supported in several studies. Shih *et al.* (Shih *et al.*, 2005) found that Nrf2 confers neuroprotection during mitochondrial stress induced by 3-nitropropionic acid (3-NP). 3-NP produces oxidative stress in the brain via multiple pathways including, but not limited to excessive mitochondrial ROS (Reynolds and Hastings, 1995; Schulz *et al.*, 1996). Mice deficient in Nrf2 activity (Nrf2 -/-) were hypersensitive to 3-NP leading to motor deficits and lesions. When mice were treated with Nrf2 activators, 3-NP toxicity was attenuated in Nrf2 +/- mice, but not in Nrf2 -/- mice. Another study indirectly supporting Nrf2's potential role in providing protection against Parkinson's

disease, was carried out by Clements *et al.* (Clements *et al.*, 2006). In this study researchers found that DJ-1/PARK7, a cancer and PD-associated protein stabilizes Nrf2 protein leading to transcription of biotransformation and antioxidant genes such as NQO1. When cells were without intact DJ-1, basal and induced transcription responses decreased. The effects of deficits in DJ-1 and impacts on Nrf2 were observed in a few other studies that found that both human and mice neuronal cells were more sensitive to toxic compounds (Yokota *et al.*, 2003; Taira *et al.*, 2004; Kim *et al.*, 2005).

6.5 Liver

With regards to Nrf2, any dysfunction of this transcription factor could have significant impacts in the liver because it regulates the basal and inducible expression of detoxifying and antioxidant genes (Yamamoto *et al.* 2004). In fact, while representing a "worst-case" scenario, a complete lack of Nrf2 activity has shown to have profound effects on liver in mice. When looking at exposures to acetaminophen (APAP) among Nrf2 knockouts, significant increases in liver injury and liver failure have been observed (Chan *et al.*, 2001; Enomoto *et al.*, 2001). Blocking the function of Nrf2 has also shown to increase ROS and lipid peroxidation in alcohol fed mice (Gong and Cederbaum, 2006).

6.6 Summary

As shown, dysfunction of the Nrf2 transcription factor could have significant effects on the expression levels of antioxidants and biotransformation enzymes including, but not limited to GSTs, NQO1, and UGTs. Although these enzymes are

not under total control by Nrf2, dysfunction of Nrf2 could potentially have greater impact than individual enzymes being that it controls the transcription of numerous genes. Despite the uncertainties regarding the impacts of Nrf2 genetic polymorphisms on disease, it is expected that a genetic polymorphism of Nrf2 leading to a less active transcription factor would increase the risk of disease. Additionally, as Nrf2 is not the only transcription factor responsible for transcription of biotransformation enzyme genes, a combination with other susceptible genotypes is likely to have more significant effects. Likewise, genetic polymorphisms leading to a more active Nrf2 protein could lead to a decrease in disease risk.

Chapter 7: Summary and Conclusions

7.1 Summary and Conclusions

With respect to the biotransformation enzymes mEH, GSTM1, GSTT1, GSTP1, and NQO1, single genetic polymorphisms, alone, are unlikely to be significant susceptibility or protective factors in the development of disease. Rather, their role as susceptibility or protective factors in disease development ultimately depends on a combination of gene-gene and gene-environment interactions, with gene-gene interaction likely playing the most significant role. In fact, no definitive or reliable information regarding disease susceptibility can be extracted from looking at single polymorphisms of mEH, GSTM1, GSTT1, and NQO1. Observations tend to be largely heterogeneous and inconsistent. Although not evaluated in this thesis, the same principle likely applies to many other polymorphisms in genes encoding other enzymes, transcription factors, transporters, antioxidants, etc.

Furthermore, even after accounting for gene-gene and gene-environment interactions, broad generalizations regarding whether a polymorphism is a risk factor is difficult. As shown in this thesis, dose, length of exposure, type of xenobiotic, diet, and other factors can be the difference between a polymorphism being a susceptible or protective factor. Differential susceptibility was observed in all of the enzymes evaluated. For example, the mEH high-activity genotype may be a susceptibility factor for smoking related lung cancer, due to increased formation of BPDE, but this same genotype may be a protective factor for smoking-related COPD where the high-activity genotype can more rapidly detoxify reactive epoxides from smoking-related

oxidative stress. Also, the NQO1 variant, conferring low-enzymatic activity was a risk factor for short-term smokers and early-onset cancer, but shows to be a protective factor in long-term smokers. In regards to dose, GSTT1 null was a risk factor for liver lesions for workers exposed to high levels of vinyl chloride in that it was unable to conjugate epoxide intermediates of vinyl chloride resulting from CYP2E1 oxidation. At low exposure levels, the GST1 non-null was a risk factor in that it bioactivated vinyl chloride. Given differential susceptibility, associations between polymorphisms and disease risk will also have to be evaluated on a chemical-specific and mechanistic basis.

7.2 Data Challenges and Uncertainties

There are numerous challenges and uncertainties with linking genetic polymorphisms with disease. With respect to the data used in this thesis, a key challenge is gathering a sampling group large enough to evaluate. A vast majority of studies that are available linking polymorphisms to disease were conducted on relatively small groups of people. In itself, this presents several additional challenges. First, large confidence limits were generated in many of the small studies; therefore, there is inherent uncertainty in the risk estimates. Second, small sampling groups made it difficult to assess gene-gene interactions that are likely more significant factors in linking genetic polymorphisms to disease. Except for the null genotypes of GSTM1 and GSTT1, many polymorphisms are only found in a small percentage of people. As a result, heterozygous genotypes had to be incorporated

into many evaluations to yield enough statistical power to draw conclusions regarding the variant alleles.

Another significant challenge and uncertainty identified in this thesis is the relative lack of data exposures, which is important when evaluating diseases tied to environmental exposures and an essential element of toxicology. Certainly the studies evaluated exposures at varying levels of detail, but the true exposure to all the different toxic substances a person is exposed to over their lifetime is relatively unknown and is nearly impossible to obtain. There is also inherent uncertainty regarding the accuracy of the exposures reported by people included in the studies.

7.3 Applicability to Regulatory Toxicology and Medicine - Future Perspectives

As discussed in the introduction, there has been a move to focus on chemical-specific susceptibility factors rather than relying on across-the-board uncertainty and safety factors. Overall, the use of genetic and genomic data will ultimately depend on its applicability to the exposures being evaluated. Currently, the use of such data will likely be more appropriate when exposures to xenobiotics, whether they are drugs or environmental pollutants, are high.

In the field of medicine, data on genetic polymorphisms and genomic-wide data will allow for a greater degree of personalized medicine and will improve drug development. Genomics data may provide clues on drug targets, which will assist in drug development (Ozdemir and Lerer, 2005). More accurate information on efficacy and safety of therapeutics will also be achieved.

In the environmental and occupational regulatory toxicology, the use of genetic polymorphism and genomics data will also be valuable. However, currently, the use of such data will likely be confined to exposures to high levels of chemicals under short-term environmental exposures and occupational exposures. Though the science is continuing to evolve, the associations between single and multiple genetic polymorphisms and risk of disease evaluated in this thesis were more evident when there were exposures to high levels of xenobiotics. At chronic low-dose environmental exposures, it is unlikely that a single or group of susceptible genotypes will impart a significant risk that is greater than the combination of uncertainty factors and/or conservatism already built into chemical-specific potency values. Adding to this is the overall complexity of associating at-risk genotypes to disease, which was shown in this thesis.

In addition to the challenges facing medicine and regulatory toxicology from a technical standpoint, the use of genetics and genomics data presents numerous social, legal, and ethical issues. Confidentiality and consent are significant concerns. The use of genetics data could hinder one's ability to obtain health insurance or seek employment. Cost and equity are also concerns (Ozdemir and Lerer, 2005). Pharmocogenetics could increase the cost of drug development with the multitude of pharmacogenetic testing that may be needed. Also, pharmacogenetics may fall short of providing adequate treatment for all individuals. Drugs may be developed for certain segments of the population where there are economic incentives to develop a drug (Ozdemir and Lerer, 2005).

7.4 Concluding Remarks

Genetics and genomics have and will continue to open the door for research and discovery in the fields of toxicology, pharmacology, medicine, etc. In regards to toxicology, the impacts of genetic polymorphisms have on the absorption, distribution, metabolism, and excretion of xenobiotics will be especially valuable. Although linking susceptible genotypes to a particular response is complex and will require a great deal more research with a genomics rather than a single gene approach; such data will be of great value when understanding potential mechanisms of toxicity and disease.

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