TOXICOGENOMIC ANALYSIS OF RISK FACTORS THAT PREDICT
SENSITIVITY TO ACETAMINOPHEN-INDUCED LIVER INJURY USING A
MOUSE MODEL OF THE HUMAN POPULATION

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A dissertation submitted to the faculty of the University of North Carolina at
Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of
Philosophy in the Curriculum in Toxicology.

Chapel Hill
2008

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ABSTRACT

ALISON HARRILL: Toxicogenomic Analysis of Risk Factors that Predict Sensitivity to Acetaminophen-Induced Liver Injury Using a Mouse Model of the Human Population
(Under the direction of Ivan Rusyn, M.D., Ph.D. and David Threadgill, Ph.D.)

Recent advances in the field of genomics have led to an improved understanding of genomic structure and function in humans and model organisms. Effective utilization of genomic information in the toxicology field has the potential to significantly improve risk assessment; however, a major limitation is a lack of animal models that can identify genetic variants underlying inter-individual differences in toxicity. Current testing strategies fail to capture sufficient genetic diversity, leading to over-generalization of the results from single-strain studies when extrapolating risk to human populations. We hypothesized that using a panel of genetically diverse inbred mouse strains (or Mouse Model of the Human Population; MMHP), would enable detection of genetic loci that affect individual toxicity responses to a model toxicant, acetaminophen. In Aim 1, we demonstrated that MMHP mouse strains experienced a range of toxicity outcomes following equal acetaminophen doses, similar to the range of toxicity observed in human subjects. Haplotype-associated mapping and genetic sequencing within the MMHP yielded a genetic variant within the gene encoding CD44 that correlated toxicity

sensitivity in both mouse and man. The results of this study indicated that use of the MMHP facilitates detection of genetic variants affecting chemical toxicity. In Aim 2, population-based biomarkers of liver injury were determined by analyzing gene expression. Identified liver injury biomarkers included several genes involved in known cell death pathways. The signature also included genes that had not been previously linked to acetaminophen-induced liver injury, indicating that the model may provide a means for discovery of novel therapeutic targets. In Aim 3, the metabolism of acetaminophen was examined across selected mouse strains. Acetaminophen toxicity requires bioactivation of acetaminophen to a quinone radical; it is therefore necessary to demonstrate whether metabolic differences potentially affect genetically pre-determined injury outcomes within the MMHP. Strain differences in acetaminophen metabolism were not a determining factor in the overall liver injury outcome, further demonstrating the need for a genetically diverse mouse model to identify therapeutic targets. Overall, the data confirm that using the Mouse Model of the Human Population as a research paradigm has the potential to improve both toxicity risk assessment and mechanistic research.

DEDICATION

To my husband, Josh, whose boundless support and love is the foundation that enabled

the building of this work; and to my parents, Ronnie Hege and Shelley Mansky, for their

constant encouragement and belief that I can achieve any goal, no matter how

daunting.

ACKNOWLEDGEMENTS

To complete a doctoral dissertation, it takes a network of support and I was extremely fortunate to have the guidance of exceptional mentors. My sincerest gratitude and appreciation is given to my mentor, Dr. Ivan Rusyn, for his support, encouragement, and invaluable guidance throughout my traineeship at UNC. My deepest thanks are given also to my co-mentor, Dr. David Threadgill, for his thoughtful advice and guidance through the course of my research. I am eternally grateful for the knowledgeable advice and recommendations contributed by Drs. Gary Boorman, Phil Smith, and Paul Watkins of my committee. Through our interactions, members of my committee constantly demonstrated a willingness to assist in traineeship and fostered an atmosphere of collaboration, of which I had the great joy to be a part. It was my deepest honor to work closely with and be mentored by each of these enthusiastic scientists.

I am indebted to the members of the Rusyn laboratory who were instrumental in assisting me not only technically, but with my growth as a scientist. In particular, I acknowledge the significant contributions of Mrs. Blair Bradford, who was always willing to lend her considerable experience to resolve my challenges. This work also was also greatly aided by the contributions of Ms. Pamela Ross, Mrs. Oksana Kosyk, Mrs.

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LIST OF ABBREVIATIONS AND SYMBOLS

α-GST	alpha glutathione-*S*-transferase
AG	acetaminophen glucuronide
ALT	alanine aminotransferase
ANOVA	analysis of variance
ANCOVA	analysis of covariance
APAP	N-acetyl-*p*-aminophenol; acetaminophen
AS	acetaminophen sulfate
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AUC	area under the curve
bp	base pairs
BP	biological process
Cyp	cytochrome P450 mixed function oxidase
D_2O	deuterium oxide
DILI	drug-induced liver injury
DNA	deoxyribonucleic acid

ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FDR	false discovery rate
GO	gene ontology
GSH	reduced glutathione
GST	glutathione-*S*-transferase
H&E	hematoxylin and eosin
HPLC	high performance liquid chromatography
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
i.g.	intra-gastric
i.p.	intra-peritoneally
KO	knockout
LDSP	Laboratory Strain Diversity Panel
LOWESS	locally weighted scatterplot smoothing
LPS	lipopolysaccharide
MDP	Mouse Diversity Panel
MF	molecular function
MMHP	Mouse Model of the Human Population

mRNA	messenger RNA
Mrp	multi-drug resistance protein
NaN$_3$	sodium azide
NADH	nicotinamide adenine dinucleotide
NAPQI	N-acetyl-*p*-benzoquinone imine
NCBI	National Center for Biotechnology Information
NOAEL	no observed adverse effect level
NMR	nuclear magnetic resonance
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PC	principal component
PCR	polymerase chain reaction
QC	quality control
QTL	quantitative trait loci
Red.	reduced
RI	recombinant inbred
RNA	ribonucleic acid
ROS	reactive oxygen species
s.c.	subcutaneous
S.E.	standard error of the mean
SNP	single nucleotide polymorphism

TSP   trimethylsilyl-propionic acid

UDP   uridine diphosphate

UNC   University of North Carolina at Chapel Hill

U.S.   United States of America

WT   wild type

Chapter I

INTRODUCTION

A. INTEGRATING STRUCTURAL AND FUNCTIONAL GENOMICS APPROACHES WITH TOXICITY TESTING TO IMPROVE RISK ASSESSMENT

Advances in genomics in recent decades have lead to exponential growth in the understanding of genome structure and function in both humans and model organisms. Due to the wealth of resources available, including databases of sequence variation and transcriptional changes following chemical exposure, both the pharmaceutical industry and agencies within the federal government have begun to invest more resources into using genomic tools to improve drug development and drug and chemical safety evaluation. Systems toxicology, or the integration of traditional toxicology approaches with the development and implementation of toxicogenomics, proteomics, and metabolomics, offers the promise of developing novel, reliable toxicity biomarkers and more accurate predictions of adverse health effects in humans.

The need for development of sensitive biomarkers to predict drug toxicity was recently reinforced by the Food and Drug Administration (FDA) Critical Path Initiative which emphasizes the development of diagnostic tests to improve clinical trials and post-market surveillance[1]. In 2005, the FDA released a final guidance document on genomic data submissions, *Guidance for Industry: Pharmacogenomic Data Submissions*[2], and announced the creation of a new FDA program entitled the Interdisciplinary Pharmacogenomic Review Group[3]. The importance of genomic information in FDA regulatory decisions is reflected in the increasing voluntary inclusion of these types of data in FDA submissions[3]. To establish reliable protocols for data

analysis and quality control in genomics studies, several consortia have been established which include academia, industry, and government agencies such as the FDA, Environmental Protection Agency, the National Institute of Standards and Technology, and the National Institutes of Health[4-6]. In the near future, a strong understanding of genomic data and its potential uses will be critical to the drug development process and clinical practice. The background information for this dissertation focuses on the current progress in the application of genomic and genetic data to drug safety and highlights recent successes and current knowledge gaps in: 1) the discovery of toxicity biomarkers or gene signatures that correlate with toxicity using **functional genomics** techniques and 2) the identification of susceptible individuals using **structural genomics** techniques. Functional genomics techniques focus on determining those changes in gene expression that are elicited by a toxic insult. Structural genomics, on the other hand, focuses on determining how alterations in genomic architecture affect toxicity phenotypes (*e.g.* genetic sequence variations that affect drug metabolism). The basis for this work is underscored by the assertion that data from each of these approaches may be used to improve understanding of toxicity mechanisms within a biological system.

B. FUNCTIONAL GENOMICS STRATEGIES TO DETERMINE TOXICITY BIOMARKERS

1.) Functional genomics allows for global gene transcript profiling of cellular toxicity responses

The major challenge of toxicology today is the ability to extrapolate risk from experimental systems to human populations. Rodents are most frequently used for *in vivo* toxicity testing, yet there often are major differences in clearance, metabolic activity of enzymes and other key biological factors between species. While it is difficult to directly translate rodent findings to human populations, human testing is most often not an option. A solution to this problem is to develop biomarkers that are: i) predictive of the toxicity response, ii) sub-clinical in that biomarkers are detectable before overt toxicity, and iii) able to be assayed from a non-invasive tissue. Toxicogenomics approaches may be used to identify gene expression patterns or signatures indicative of adverse health events at low doses. However, to accomplish these goals, the gene expression changes (and associated pathways) that are best associated with impending pathology must be identified and separated from benign adaptive changes that are responsive to the chemical but are not associated with toxicity.

At the forefront of these emerging technologies is the use of **functional genomics** or analysis of molecular perturbations as measured by transcriptomics and assayed by microarray technology. Gene expression analysis using current microarray platforms that encompass whole genomes allows for a comprehensive picture of cellular responses[7], especially when examined in concert with proteomic and metabolomic data. While early microarray experiments were often criticized as "fishing

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expeditions", current testing strategies enable the generation of testable hypotheses that aid in understanding the mechanism of toxicity[8]. The ultimate value of using transcript profiling data within the context of systems toxicology is that responses that are predictive of cellular injury can be sampled across doses, time points, and species, facilitating risk extrapolation to human populations.

2.) Improving safety assessment through genomic profiling

Microarray profiling has become a major tool for the characterization of drug toxicities by allowing for large-scale determination of gene expression changes associated with a defined pathology. A key step in toxicogenomics is phenotypic anchoring, defined as the ability to link a chemically-elicited phenotype with gene expression changes[9]. Experimentally determined gene expression signatures can serve as a guide for determining biomarkers that are indicative of toxicological responses that may be as-yet sub-clinical, with no observable morphological changes[10]. To confirm the utility of the approach, Heinloth et al.[11] demonstrated that patterns of gene expression perturbations observed at sub-toxic doses of acetaminophen in rats may indicate subtle cellular injury that was not detectable by overt pathology or clinical chemistry parameters within the liver. At toxic doses, expression changes in the same subset of genes associated with mitochondrial dysfunction and oxidative stress were more exaggerated and changes were detected in additional genes associated with these processes. These

data indicate that gene expression profiling has the potential to identify subtle markers of cellular injury that precipitate overt organ toxicity.

3.) Identification of biomarkers of toxicity

Identification of sensitive biomarkers that will assist in monitoring drug therapy for evidence of toxicity or therapeutic outcome, and (in acute poisoning cases) to predict exposure levels, is a critical area where omics technologies can be applied. Genomic biomarkers of toxicity have recently been identified for a wide variety of toxicants including nephrotoxic agents[12], testicular toxicants[13], and for keratinocyte proliferation in papilloma murine skin model[14], to name only a few. The potential for using this technology to identify safety biomarkers is great and may help to create better diagnostic tools for the traditionally difficult task of toxicodynamic monitoring, such as in patients receiving immunosuppressive therapy[15].

The majority of recent investigations have used microarrays to study toxicity in target organ tissue or in cultured cells. While these experiments often yield important insight into the mechanism of toxicity, they provide limited information for monitoring drug safety in patient populations through non-invasive means. To address this limitation, Bushel et al.[16] investigated the utility of measuring gene expression signatures in peripheral blood as an indicator of pathological changes in the liver following administration of varying doses of acetaminophen. In this study, a prediction algorithm

using liver injury classifiers and a pattern-based method that was weighted toward non-injurious exposure levels was used to discriminate sub-toxic and toxic exposure doses. Characterization of acetaminophen-induced liver injury using gene expression profiles derived from blood was shown to better predict acute chemical exposure levels than clinical chemistry, hematology, or histopathology analysis, indicating that transcript profiles derived from blood may be a good marker for specific organ toxicity.

4.) Pathway analysis may facilitate mode of action determination

A major knowledge gap in connecting gene expression profiles to classes of toxicants that elicit similar phenotypes is a lack of complete information regarding the complexity of cellular molecular pathways. Regulation of responses can be relatively straightforward (i.e., at the transcriptional level), but it can also be quite complex and controlled by multiple genes, proteins, and metabolites. Therefore, accurate identification of gene-gene interactions and regulation is essential for determining well-defined pathways that could serve as potential targets in therapeutic development or intervention. The importance of understanding subtle pathway changes and applying the data to risk assessment has been underlined in the National Research Council's report entitled "Toxicity Testing in the 21st Century"[17]. Transcriptional pathway-level analysis may prove essential for identifying molecular perturbations that are associated with sub-clinical pathologies.

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There are several public databases available which aid in the annotation and interpretation of gene expression data in terms of cellular process, functions, and pathways. These databases include the Gene Ontology (GO; www.geneontology.org), Gene Map Annotator and Pathway Profiler (www.genmapp.org), the Science Signaling Connections Map (stke.sciencemag.org/cm/), BioCarta (www.biocarta.com/genes/index.asp), Reactome (www.genomeknowledge.org), KEGG (www.genome.jp/kegg/pathway.html), and Ingenuity (www.ingenuity.com). Most often, these tools are used to associate a set of differentially expressed genes from a microarray experiment with a particular pathway with the goal of identifying key modes of action. Several groups have developed statistical methods for associating annotated cellular pathways with gene expression changes such as GOMiner[18], Significance Analysis of Function and Expression (SAFE)[19], and Onto-Tools[20]. While the methods employed by these statistical techniques vary, each is aimed at assigning biological meaning to gene expression data. A broad understanding of the pathways affected by a variety of xenobiotics is essential for the future of drug safety assessment. A complete understanding of the molecular perturbations that precipitate a toxic pathology *in vivo* will enable a shift from high dose to low dose testing and may enable a better prediction of pathology from *in vitro* and *in vivo* data when similar patterns of perturbations are observed.

C. STRUCTURAL GENOMICS APPROACHES TO DETERMINE SUSCEPTIBLE INDIVIDUALS

1.) Identifying sensitive individuals

Toxicogenetics is a discipline that evaluates genetic sequence variations that may impact individual's susceptibility to toxicity. In the pharmaceutical arena, these efforts are largely aimed at identification of susceptible individuals within a prospective patient population, thereby enabling personalized drug treatment and improved drug safety. It is well accepted that genetic variants affect responses to drugs[21, 22]. One of the first studies in this field compared plasma drug half-lives in identical and fraternal twin pairs and showed that greater differences existed between fraternal twins[23]. Recently, several more monogenic toxicogenetic traits have been reported[24]. Such research has yielded some success into the identification of genetic alleles that predict drug responses. For example it has been demonstrated that *apolipoprotein E* (*APOE*)-4/4 allele carriers are the worst responders to conventional Alzheimer's disease treatments[25].

Successes in this field have led to important regulatory action by the FDA that has allowed a number of drugs to remain on the market due to the availability of genetic testing. Current genetic tests include assigning the dose of 6-mercaptopurine based on the genotype of thiopurine *S*-methyltransferase (*TPMT*)[26, 27], and the dose of warfarin based on the genotypes of vitamin K epoxide reductase complex (*VKORC1*)[28] and *CYP2C9*[29]. In the case of the anti-coagulant drug warfarin, the combination of *VKORC1*

haplotyping and *CYP2C9* genotyping explained an estimated 25% and 6-10% of the variance in warfarin dose, respectively[30]. In fact, warfarin represents one of the first drugs for which toxicogenetic information (by VKORC1 haplotyping) better explained the dose variance than pharmacokinetic pharmacogenomic data (through *CYP2C9* genotyping)[31]. The data on warfarin, therefore, represents a shift from monogenic toxicogenetic testing to a polygenic model, which can be expected to be utilized with increasing frequency as research in this field expands to include a greater variety of pharmaceuticals.

The wealth of information on genetic polymorphisms now available through the Human Genome Project has led to a dramatic increase in studies that seek to connect genetic variants with toxicity and pharmacologic phenotypes. Because the base pair sequence variation among individuals averages to be about 1 in 500-1000 base pairs[32], it is reasonable to expect that a significant number of genes will contain polymorphisms that contribute to disease and that many will play a role in adverse drug responses. Most of the current research focuses on the association between phenotype/disease and single nucleotide polymorphisms (SNPs). SNPs are an attractive choice for biomarkers of adverse responses because, unlike other factors which contribute to a toxicity phenotype such as age, co-morbidity, and environment, an individual's genetic code remains stable throughout their lifetime. Genetic testing offers the potential of replacing empirical dose adjustment for many drugs that is based upon therapeutic

assessment of pharmacologic or toxic effect after initial dosing. In addition, predictive

genetic tests could also be of value in the drug development process by rescuing drugs

that failed Phase III clinical trials due to toxicity within a subset of participants[33]. A key

example of this is the genetic testing available to patients with HIV who are prescribed

the drug abacavir, in which screening for major histocompatibility complex, class I, B

(HLA-B)*5701 reduces the risk of hypersensitivity reactions[34].

2.) Toxicogenetic models and approaches

A number of publications have reported a significant association between SNPs and

disease phenotypes in many areas of research; however few have been validated and

these studies have often been followed by reports that refute the original conclusion[35].

Reasons for discrepancies between genotype-and-phenotype are numerous, but are

often due to a low sample size in the study, population stratification of alleles, and

heterogeneity of phenotypic classification[36, 37]. To better facilitate a clinical translation of

toxicogenetic data, efficient and validated strategies are needed. Recent successes in

this research area include clinical data that has been used to determine the genetic

variation underlying complex traits such as Parkinson disease[38] and susceptibility to HIV

infection[39].

Classical approaches in this area have focused on mapping quantitative trait loci

(QTL) within the genome that influence a specific phenotype. While there are many

approaches to identify QTL, all involve a population of individuals with a measurable phenotype, a database of genotypic variation present within that population, and statistical measures that serve to link the magnitude of the phenotype with a specific genotype or polymorphism[40]. Classical approaches have often sought to utilize the genotypic and phenotypic diversity present in F2 or backcross mouse populations; however, this approach is limited by the necessity to genotype all individuals within a population. Due to the relatively low number of recombination events in F2 and backcross populations, identification of precise QTL locations is often more difficult. Recombinant inbred lines offer the advantage of fixed genomes, but these lines can be expensive to acquire and maintain.

A promising new alternative is a method known as **genomic association mapping** in classical inbred mouse lines, which takes advantage of the genetic variation that arose naturally across inbred mouse strains over decades of crosses and inbreeding by scientists and fanciers. In this approach, large single nucleotide polymorphism (SNP) datasets for several dozen strains and a database of over 8 million SNPs for 15 inbred mouse strains have recently become publicly available[41]. Computational methods for genomic association mapping using inbred mouse strains have been recently described[40, 42], enabling the use of these approaches to determining genetic sequence variants that affect toxicity responses.

Applications of genomic association algorithms to toxicology have been performed *in vivo* within inbred mouse strains to determine additional genetic factors that affect the metabolism of warfarin[43]. The metabolism of warfarin, specifically the generation of 7-hydroxywarfarin, was shown to vary across inbred strains. This phenotype was then computationally associated with the mouse genomic region that encodes for *Cyp2c* family enzymes. Experimental validation narrowed the list of potential genomic candidates to show that *Cyp2c29* polymorphisms altered hepatic protein expression of this enzyme. In a subsequent publication[44], this same group demonstrated the utility of this approach in an *in vitro* drug biotransformation system in which genomic association was performed for downstream metabolism of testosterone and irinotecan across 15 mouse lines. The results of these studies showed that genetic variation within the *Cyp2b9* and *Ugt1a* loci influenced the metabolism of α-hydroxytestosterone[43] and irinotecan glucuronidation[44], respectively. These results were then confirmed experimentally using recombinant enzymes. These results suggest that genomic association using inbred mouse strains has the potential to aid in the identification of genetic alleles that play a role in toxicity responses. However, further studies that demonstrate a clear translation to human populations is required.

D. INTEGRATION OF OMICS DATA WITH TRADITIONAL TOXICOLOGY

The need to better describe biological systems has led to the study of systems toxicology. Systems toxicology comprises the integration of genetics, metabolomics, and conventional toxicity endpoints into a systems biology approach. These integrative approaches often use mouse models for: i) an improved characterization of toxicity pathways, ii) the discovery of new molecular and cellular indicators of exposure and outcome, iii) better dose-response assessment, and iv) improved inter-individual/cross-species extrapolations (Figure 1.1).

While microarray-based approaches in toxicity studies generate a wealth of data on gene expression and pathways that are affected by treatment or that are associated with a particular phenotype, these data are most often descriptive and may not reflect changes at the protein level. A key limitation of an analysis of toxicogenomic data alone is that these data often do not take into account confounding factors such as the pharmacokinetics of the test chemical[45] and environmental factors, such the gut microflora population[46]. Genomic studies, while more comprehensive than high-throughput metabolite or protein analysis, fail to characterize the full complement of cellular proteins which are subject to post-translational modifications and additional regulation[47]. To address this concern and to develop additional toxicity biomarkers, it is important to consider the pharmacokinetics of the test chemical *in vivo* because genomic approaches, when analyzed alone, are limited in their ability to completely

describe dose kinetics and post-transcriptional biological complexity present within biological systems.

Databases and approaches are currently being developed that can establish links between genomic, metabolomic, and proteomic data with the goal of placing toxicogenomics data into a larger biological perspective. These include the Comparative Toxicogenomics Database (ctd.mdibl.org), the Distributed Structure-Searchable Toxicity database (www.epa.gov/ncct/dsstox/index.html), the Critical Path Institute (www.c-path.org), and systems biology databases developed by GeneLogic, Inc., (www.genelogic.com), Iconix Pharmaceuticals (www.iconixbiosciences.com) and Ceetox, (www.ceetox.com). As more data is added to publicly- and privately-funded databases, the data can be re-used and integrated to better inform hypothesis-driven research[48]. It is important to collect and archive data for a wide variety of compounds across multiple genotypes in order to understand the basis for individual differences in drug response.

The **overall goal** of the present series of studies is to develop a rodent model that facilitates determination of toxicity biomarkers across a genetically heterogeneous population. To accomplish this goal, we integrate functional and structural genomics approaches with "classical" toxicological assessments of pathology, clinical chemistry, and pharmacokinetics in order to assess the validity of the approach to translate findings to human populations.

E. RATIONALE AND SPECIFIC AIMS

1.) Drug-induced liver injury is a significant public health concern

Drug-induced liver injury (DILI) is the most prominent reason for cessation of pharmaceutical testing in clinical trials, limitations on drug use, and the withdrawal of approved drugs[49]; recent data from the United States Acute Liver Failure Study Group indicate that DILI accounts for more than 50% of cases of liver failure[50]. Adverse hepatic drug reactions remain a significant safety concern because they are often idiosyncratic in nature and occur at rates that are too low to be detected in standard-sized clinical trials[51]. Because of the relatively low frequency of occurrence during drug testing, drug-induced liver injury is often difficult to anticipate and prevent.

There are currently no diagnostic tests available to clinicians to pre-screen patients for DILI. Factors that contribute to DILI in a single individual are various, and often require contributions from genetics to environmental variables and lifestyle habits. In pharmaceutical toxicity testing, the dogmatic approach is to screen a chemical against a single outbred rodent strain, in which every mouse must be genotyped in order to look for genetic variations that affect toxicity phenotypes. This approach is limited in its ability to detect genetic polymorphisms that influence mechanisms of hepatotoxicity in humans and larger numbers of rodents need to be screened before an effect can be considered statistically significant. Therefore, better pre-clinical models are

required that can accurately predict and identify genetic variants that predispose individuals within a heterogeneous population to drug toxicity from pharmaceutical agents.

2.) Acetaminophen-induced liver injury has a complex etiology

In these studies, we investigate genetic causes of variation in the hepatotoxicity of the commonly used analgesic drug, acetaminophen (N-acetyl-*p*-aminophenol; APAP; Tylenol™). In the United States, 39% of cases of DILI are due to overdose of APAP, either intentionally or by "therapeutic misadventure", and 13% are due to idiosyncratic liver injury associated with other drugs[50]. APAP has not typically been considered an idiosyncratic drug because overdose is known to cause acute toxicity within the liver. However, a considerable number of participants in a recent clinical trial who were administered a daily intake of the maximum therapeutic dose of APAP (4 g/day in four equal doses) experienced elevations in serum alanine aminotransferase (ALT) levels that were greater than three times the upper limit of normal[52], indicative of liver injury. Additionally, while more than one third of all cases involving acute liver failure in the United States are due to APAP overdose[53], about half of these cases are unintentional and involve chronic ingestion of sub-acute doses[54].

Liver injury due to acetaminophen overdose represents a complex phenotype, requiring accumulation of its reactive metabolite, N-acetyl-*p*-benzoquinone imine

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(NAPQI), and the depletion of reduced glutathione (GSH) within the liver. Processes that have been shown to play a role in the mechanism leading to injury progression include covalent binding to cellular proteins, oxidative stress, apoptosis, necrosis, and disruption of calcium homeostasis (Figure 1.2). Following oxidative stress events caused by acetaminophen metabolism, aggravation of liver injury has been linked to activation of the innate immune system[55, 56], including Kupffer cell activation and an imbalance between protective and injurious cytokines[57-59]. The recruitment of neutrophils to pericentral regions has also been implicated as a risk factor for increased injury[56,60], but much debate exists on their importance in the liver injury outcome[61-63]. While much recent research on APAP-induced liver injury has focused on the specific contributions of innate immunity, the precise mechanisms by which injury continues after the drug has been fully metabolized remain unclear. Due to the complexity of factors contributing to APAP-induced toxicity, it has been difficult to determine the underlying causes that might predispose any specific individual to drug-induced liver injury. The development of a mouse model that can identify inherent susceptibility factors is an important first step toward predicting and preventing rare hepatotoxic reactions.

3.) Design of the Mouse Model of the Human Population

There is a greater ability to detect genetic variants that contribute to a phenotype when utilizing multiple inbred strains rather than a single outbred strain. In toxicity

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studies, most phenotypic outcomes have a polygenic mode of inheritance in which the phenotypic variance is defined as the sum of the genetic variance and the environmental variance. Because all mice within an inbred strain are identical, the genetic variance is zero and the phenotypic variance is thus equal to the environmental variance. In contrast, in an outbred stock (in which the genetic variance is greater than zero), the phenotypic variance will be much greater and the detection of an effect will require significantly larger numbers of animals per study, although the extent will depend on the heritability of the trait and the degree of genetic variation present within the outbred colony. For these major reasons, some experts recommend using a "factorial" design for experimental detection of toxicity in which the number of mice needed to detect an effect, as designated by statistical power calculations, can be split among multiple strains of equal groups[57]. Such an approach would have increased value over using either an outbred or a single inbred strain because the experiment retains statistical power, can offer insight into whether the toxicity response is influenced by strain genetics, and overcomes the problem of resistance within a particular strain obscuring the effect. In addition, testing in inbred mouse strains facilitates genomic mapping of loci that contribute to a particular toxicity phenotype due to the large SNP databases available for many commonly used strains[40].

4.) Specific aims of the research

The diversity of genotypes among different mouse strains is suitable for studying genetic components that influence pathological outcome because it has been demonstrated that the genetic diversity among mouse strains is as great as that within the human population[64]. Genomics strategies using mouse diversity panels that have thus far have been utilized to understand and predict individual drug responses have been limited by a lack of human validation. There is additionally a lack of information on genetic variants that affect acetaminophen-induced liver injury. *Therefore, we hypothesized that, by combining classical toxicological endpoints with an extensive knowledge base of rodent genetics, we could effectively model human genetic sensitivity to acetaminophen-induced liver injury by using a panel of genetically diverse inbred mouse strains.* To test this hypothesis, we proposed three specific aims designed to evaluate the Mouse Model of the Human Population (MMHP) and assess the ability of the model to discover novel biomarkers of toxicity response and effect within genetically different individuals.

Specific Aim 1. In a recent clinical trial conducted by our collaborators at UNC and at Purdue Pharma, it was shown that more than 50% of human volunteers taking the maximum dose of APAP over a seven day in-clinic study developed significantly elevated serum ALT levels[52]. A similar exposure study was performed using a second

human cohort at UNC, thereby replicating the results in which some individuals experience ALT elevations while others remain at control levels. The study provided a unique opportunity to test whether differential toxicity responses observed in humans could be replicated within a mouse diversity panel. In addition, because genomic DNA from both humans and mice were available, we were afforded a unique opportunity to validate potential toxicogenomic loci determined by haplotype-associated mapping in mice. We hypothesized that human subject sensitivity to APAP-induced liver injury is due to genetic polymorphisms that can be identified in the genetically diverse mouse strain panel. Therefore, in *Aim* 1, we sought to validate the MMHP by assessing whether genetically-determined hepatotoxicity following an acute dose of APAP reflects the range of hepatotoxicity observed in human cohorts following APAP administration. In addition, we sought to determine the functional consequences of potential toxicity-modulating polymorphisms on the APAP-toxicity outcome using gene knock-out mice and *in silico* protein predictions.

Specific Aim 2. The mechanisms by which APAP contributes to DILI have been extensively studied; however, much debate remains on the specific contributions of metabolic processes, intracellular signaling, and extracellular signaling events following acute APAP doses, given that N-acetylcysteine administration is an effective early antidote. As a result of *Aim 1*, we demonstrated that there were diverse strain-specific

liver injury outcomes across the MMHP. High-throughput microarray studies are increasingly applied to the discovery of potential biomarkers of adverse health events; however, data obtained from studies using a single strain should be interpreted with some caution because biomarkers may be strain-specific and therefore may not be useful to assess responses within heterogeneous human populations. We hypothesized that that liver gene expression profiling would yield a set of liver injury "population-based" biomarkers. In *Aim 2*, we performed global gene expression profiling derived from the MMHP following an acute dose of APAP. Using microarrays and an endpoint used traditionally to assess hepatotoxicity (*i.e.* the percent liver necrosis), we sought to determine a set of genes for which expression correlates with the liver injury outcome, but is independent of mouse strain.

Specific Aim 3. Due to the complexity of factors required to initiate and propagate APAP-induced liver injury, it is important to determine which key processes are affected by differences in genetics. In *Aim 1* and *Aim 2*, we used *in silico* and high-throughput gene expression profiling methods to determine biomarkers of sensitivity and injury response. However, the samples used to generate these data were derived from tissues extracted temporally downstream of APAP metabolism in the mouse. Therefore, we previously were unable to determine whether there are strain-specific differences in the metabolic bioactivation of APAP to its reactive, injury-causing metabolite. We

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hypothesized that potential differences in APAP metabolism, while important to initiating toxicity, do not determine the overall liver injury outcome within the MMHP. Therefore, in *Aim 3*, we investigated the pharmacokinetics of APAP within five strains of varying sensitivity to APAP-induced liver injury and assessed the relevance for potential strain-dependent metabolic differences to affect the overall toxicity outcome.

Figure 1.1

Information flow in systems toxicology
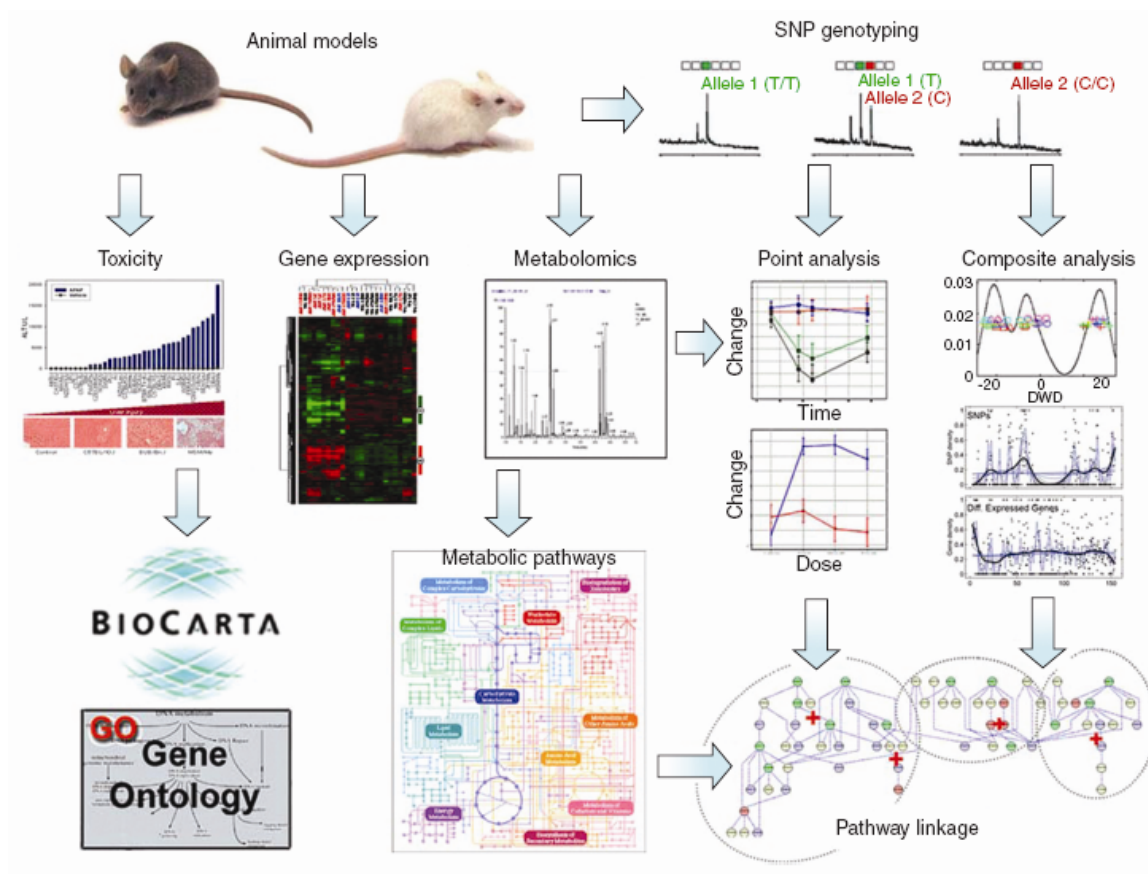
Figure 1.2

Factors contributing to acetaminophen-induced liver injury

Hepatotoxicity due to an overdose of APAP is initiated by metabolic events that involve the bioactivation of APAP to a reactive quinone, NAPQI, that binds to key hepatocellular proteins. Stressed hepatocytes initiate a signaling cascade that can result in the activation of innate immune cells that can affect the propagation of the liver injury response. Adapted from Kaplowitz[54].

Abbreviations: acetaminophen, APAP; acetaminophen glucuronide, APAP-G; acetaminophen sulfate, APAP-S; Cytochrome P450 2E1, Cyp2e1; N-acetyl-p-benzoquinoneimine, NAPQI; reduced glutathione, GSH; Gluathione *S*-transferase, GST; reactive oxygen species, ROS; Acetaminophen glutathione, NAPQI-SG; adenosine triphosphate, ATP; lipopolysaccharide, LPS; liver sinusoidal epithelial cells, LSEC; Fas ligand, FasL.
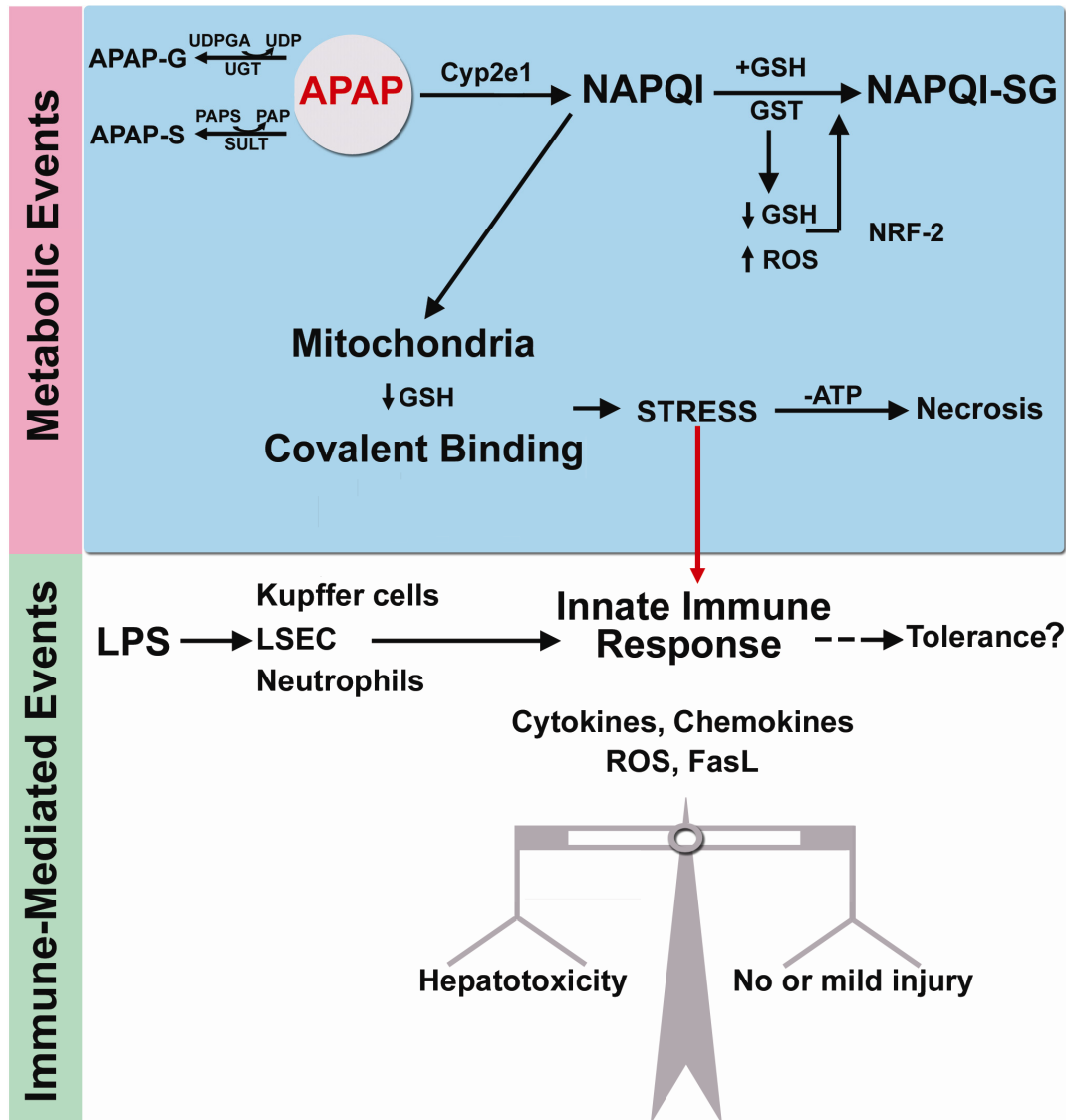
Chapter 2

MOUSE MODEL OF THE HUMAN POPULATION REVEALS THAT VARIANTS IN
CD44 CONTRIBUTE TO ACETAMINOPHEN-INDUCED LIVER INJURY IN HUMANS

A. ABSTRACT

Inter-individual variability in response to chemicals and drugs is a common regulatory concern. It is assumed that xenobiotic-induced adverse reactions have a strong genetic basis, but many mechanism-based investigations have not been successful in identifying susceptible individuals. While recent advances in pharmacogenetics of adverse drug reactions show promise, the small size of the populations susceptible to important adverse events limits the utility of whole-genome association studies conducted entirely in humans. We present a novel strategy to identify genetic polymorphisms that may underlie susceptibility to adverse drug reactions. First, in a cohort of healthy adults who received the maximum recommended dose of acetaminophen (4 g/day X 7 days), we confirm that about one third of subjects develop elevations in serum alanine aminotransferase indicative of liver injury. To identify the genetic basis for this susceptibility, a panel of 36 inbred mouse strains was used to model human genetic diversity. Mice were treated with 300 mg/kg acetaminophen and the extent of liver injury quantified. We then employed whole-genome association analysis and targeted sequencing to determine that polymorphisms in *Ly86*, *Cd44*, *Cd59a*, and *Capn8* correlate strongly with liver injury. Finally, we demonstrated that variation in the orthologous human gene, *CD44*, is associated with susceptibility to acetaminophen in two independent cohorts. Our results indicate a role for CD44 in modulation of susceptibility to acetaminophen hepatotoxicity. These studies

demonstrate that a diverse mouse population can be used to understand and predict adverse toxicity in heterogeneous human populations.

B. INTRODUCTION

Adverse reactions, such as liver injury, are prominent reasons for cessation of drug testing in clinical trials, restrictions on drug use, and the withdrawal of approved drugs[65]. Adverse reactions remain a significant safety concern since they occur at low rates, often undetectable in standard-sized clinical trials, and are not foreseen through traditional *in vitro* and animal safety testing paradigms[66]. While it is widely recognized that better pre-clinical models are required to enable accurate prediction and identification of xenobiotic-induced toxicity[67], few experimental paradigms exist that provide preclinical population-wide testing.

The promise of personalized medicine and the accumulating knowledge of human genomic variation serve as potent catalysts for pharmacogenetics research[68]. Polymorphisms within genes encoding xenobiotic metabolizing enzymes and major histocompatibility complex proteins are promising genetic biomarkers that may predict the efficacy of drug treatment or identify individuals at risk of adverse reactions[69, 70]. However, only a limited number of potentially useful biomarkers have been identified thus far. Furthermore, current research into pharmacogenetic biomarkers is largely focused on human studies where only a limited number of positive associations

between a polymorphism and adverse drug reaction have been reproduced in independent cohorts.

For the past century, the mouse has been the most widely used model system for studying human disease and related phenotypes, often in ways that are not directly possible in humans [71]. Many laboratory studies take advantage of the fact that the genomes of inbred strains are a mosaic of regions that are derived from different subspecies of *Mus musculus*[72]. The major mouse genetic resource used for association studies of complex polygenic traits is the Laboratory Strain Diversity Panel (LSDP)[73]. Recent resequencing of 15 mouse inbred strains and the analysis of their polymorphism architecture[74] have shown that an LSDP contains as many or more single nucleotide polymorphisms (SNPs) than estimated to be present in humans, and minor allele frequency distribution in the LSDP is largely similar to that present in man. Thus, we hypothesized that a panel of inbred mouse strains (or mouse model of the human population; MMHP) can be used to model the diverse human population and to uncover susceptibility factors for drug-induced toxicities, thus shortening the path to the discovery of pharmacogenetic biomarkers.

In this study, we tested this novel approach by investigating the genetic causes of variation in the hepatotoxicity of acetaminophen (N-acetyl-*p*-aminophenol). More than a third of all cases involving acute liver failure in the United States are due to overdose of this widely available medication[75]; about half of these cases are unintentional or

involve chronic ingestion[54]. In addition, it has been estimated that 10% of patients experiencing liver failure due to acetaminophen were taking recommended doses of acetaminophen[76]. Liver injury due to acetaminophen is a complex phenotype, requiring accumulation of its reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), covalent binding to cellular proteins, oxidative stress, and hepatocellular necrosis, as well as an imbalance between protective and injurious cytokines[77, 78]. A recent placebo-controlled clinical study revealed that about a third of healthy adult volunteers who were administered the maximum therapeutic dose of acetaminophen (4 g/day for 14 days) exhibited transient, asymptomatic elevations in serum alanine aminotransferase (ALT) levels that were greater than three times the upper limit of normal[52], indicating liver toxicity. Acetaminophen represents an intriguing model compound for pharmacogenetic studies, because, while subjects taking therapeutic doses of the drug exhibit transient serum ALT elevations, the drug has a good safety profile in long-term use[79]. The same pharmacogenetic factors that predispose a person to transient low-dose ALT elevations may be responsible for decreasing that individual's hepatotoxic susceptibility threshold at higher doses. For these reasons, acetaminophen is an ideal compound for the validation of a human-to-mouse-to-human approach in pharmacogenetic research.

C. METHODS

Acetaminophen Administration to Human Subjects

Study volunteers were healthy men and women between 18-45 years of age, and not receiving concomitant medications. Prescreening was performed 14 days prior to admission to confirm health as previously described[52]. Written informed consent was obtained and approved by the UNC Institutional Review Board. Participants remained in the General Clinical Research Center at UNC for the duration of the 14 day study during which they received a controlled diet of standardized whole-food meals. From days 4 to 11, subjects received either Extra Strength Tylenol (two 500 mg tablets of acetaminophen, commercial product; n=49) or placebo (n=10) orally every 6 hours. Blood samples were taken at 8 am daily prior to dosing and analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT; Appendix 1), total bilirubin, alkaline phosphatase, blood urea nitrogen, glutathione alpha-*S*-transferase, and creatinine. Dosing was discontinued for subjects in whom serum ALT or AST reached more than 3 times upper limit of normal. Baseline serum ALT was determined as the mean of the values obtained prior to the start of dosing. Blood was collected from study participants for DNA isolation. Leukocytes were isolated from whole blood and DNA was extracted using the Qiagen MidiPrep kit (Qiagen) and the manufacturer's protocol. The protocol for the Purdue Pharma L.P. cohort study has been as previously described[52].

Acetaminophen Administration to Mice

Toxicity Studies. Male mice aged 7-9 wks were obtained from Jackson Laboratory and housed in polycarbonate cages on Sani-Chips irradiated hardwood bedding (P.J. Murphy Forest Products Corp). Animals were fed NTP-2000 diet (Zeigler Brothers, Inc.) and water *ad libitum*, and maintained on a 12 h light-dark cycle. Mice utilized in this study comprise 36 inbred strains that are priority strains for the Mouse Phenome Project[80]; B6C3F1/J hybrid mice were also used. Care of mice followed institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee. Mice were singly housed and fasted 18 h prior to intra-gastric dosing with acetaminophen (30, 100, 300, 600, 900, or 1200 mg/kg; 99% pure, Sigma-Aldrich) or vehicle (0.5% methyl 2-hydroxyethyl cellulose, Sigma-Aldrich) with a dosing volume of 10 ml/kg for all doses. Dosing was performed at the same time of day throughout the study to avoid diurnal variability[81]. Feed was returned 3 h after dosing and animals were sacrificed at 4 or 24 h. Blood was collected from the vena cava from animals anesthetized with nembutal (100 mg/kg *i.p.*, Abott Laboratories). Samples were assayed for serum markers by standard enzymatic procedures[82]. Livers were quickly excised and sections of the left lobes were placed in 10% phosphate buffered formalin for immunohistochemical analyses. Remaining liver was snap-frozen in liquid nitrogen and stored at -80ºC.

Metabolism Studies. Adult (aged 7 weeks) male mice of strains C3H/HeJ, C57BL/6J, DBA/2J, LP/J, and NZW/LacJ were selected for metabolism studies based on their wide variation of liver toxicity observed at 24 h following a 300 mg/kg APAP dose. Mice were fed overnight prior to dosing with 50 mg/kg APAP or fasted for 18 h overnight prior to dosing with 300 mg/kg APAP (N=5 per strain per dose). Blood (45 µl) was collected sequentially from the tail vein at 0, 0.5, 1, 2, and 3 h post-dosing. At 6 h, blood was collected by exsanguination at 6 h for metabolite measurements and ALT quantification and livers collected as described above.

CD44 Knockout Studies. To test the ability of CD44 protein to modulate APAP toxicity, CD44 knockout mice, B6.Cg-Cd44[tm1Hbg]/J (N=6), and wild-type mice, C57BL/6J (N=6), were dosed with 300 mg/kg APAP (*i.g.)* and sacrificed at 24 h as described in toxicity studies. An interim blood sample was collected from the tail vein at 4 h post-dosing for ALT analysis.

Glutathione Quantification

Liver samples were homogenized in borax/EDTA (pH 9.3) solution, precipitated with chloroform and centrifuged. Reduced glutathione was derived in liver samples, calibration standards, and QC samples with 7-fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-F) and analyzed by HPLC with fluorescence detection. Concentrations were calculated using the glutathione response, sample weights, and a regression line constructed from the concentrations and peak responses of the

appropriate calibration standards (Sigma). Glutathione detection assays were performed by Battelle (Columbus, OH).

Enzyme-linked Immunosorbent Assay (ELISA)

Quantitative determinations of protein levels of cytochrome p450(CYP) 2E1, CYP1A2, catalase, and glutathione S-transferase (GST) was performed using microsomes isolated from the left liver lobe using the Protein Detector ELISA kit protocol (KPL, Inc. Gaithersburg, MD) as detailed by the manufacturer. The ELISAs were performed by Integrated Laboratory Systems, Inc. (Research Triangle Park, NC).

Liver Histopathology

Formalin-fixed liver specimens were embedded in paraffin and 5 μm sections cut in duplicate were applied to each slide. Sections were stained with hematoxylin and eosin (H&E) and liver injury was blindly scored. Necrosis was quantified by a point counting technique[83]. Scores were independently verified by a veterinary pathologist.

Serum Metabolite Quantification

The procedure used for the quantification of APAP is similar to that previously described[84]. Briefly, a reversed-phase HPLC assay was used in which the mobile phase was 5% acetonitrile and 95% 5 mM sodium sulfate/20 mM potassium phosphate buffer (pH=3.2) with a flow rate of 1.2 ml/min. Retention times for APAP and the internal standard (3-acetamidophenol) detected at 254 nm were 7 and 11 min respectively. APAP standard (Sigma Chemical, St. Louis, MO) and the internal standard were spiked

into naive mouse plasma to generate standard curves. The AUC was calculated by using noncompartmental analysis in WinNonLin (Pharsight, Mountain View, CA). A one-way ANOVA with a Tukey post-hoc test was used to assess significantly different AUC across mouse strains ($P<0.05$).

Haplotype Association Mapping

Haplotype association mapping was performed as described elsewhere[85]. Briefly, haplotype associations were calculated using a modified F-statistic based upon genotype-phenotype pairings at each 3-SNP window across a 160,000 SNP dataset. Strains excluded from association analysis due to lack of polymorphism data were C57BL/10J, NZO/H1LtJ, and P/J. Log$P$ values were plotted across the mouse genome using SpotFire (SpotFire, Inc.). Genomic intervals with association scores greater than 3.5 were considered significant. Genes within significant intervals were identified with the BioMart feature of Ensembl using NCBI build 36 (http://www.ensembl.org).

Genetic Sequence Analysis

For sequence-based genotyping, primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For each reaction, genomic DNA from pedigreed mice or from human subjects was diluted to 100 ng/µl and 1 µl of DNA mixed with 12.5 µl of 2x PCR Master Mix (Promega), 2.5 µl of 10 µM upstream primer, 2.5 µl of 10 µM downstream primer and 6.5 µl of nuclease-free water. Primers and conditions used for PCR amplification are listed in Appendix 1. Sequencing

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reactions were performed using the ABI PRISM™ BigDye™ Terminator Version 1.1 Cycle Sequencing Ready Reaction Kit with the AmpliTaq^R DNA Polymerase (Applied Biosystems). DNA was sequenced on a 3730 DNA Analyzer (Applied Biosystems) (Appendix 2). Sequence alignment was performed using Vector NTI version 10 (Invitrogen).

Statistical Methods

Phenotypic values were expressed as the mean ± standard error of the mean. Differences were considered significant when the *P*-value < 0.05. The Pearson correlation coefficient was used to determine correlation between phenotypic toxicity measurements. Genotype-to-phenotype associations for the mouse data were performed using the two-tailed Student's t-test (for two variants) or ANOVA (for more than two variants). *P*-values were adjusted for multiple comparisons using a false discovery rate of 5% for the total number of SNPs genotyped in mouse strains across the six genes. *P*-value corrections were performed using the p.adjust module in R (v. 2.4.0). Correlation between human genotype data for *CD44* and phenotypic responses across time was performed in Partek Genomics Suite (Partek) using repeated measures ANOVA across the first seven days of acetaminophen treatment in which the study centers were coded as random effects (Appendix 3). In determining the effect of genotype to influence serum ALT increases in acetaminophen-treated human subjects during treatment at UNC, we excluded subjects whose average baseline was 55 U/L or

greater (4 subjects). Elevations in ALT level within each subject were analyzed using linear modeling in which the daily ALT of each individual over time was assigned a p-value using lm{stats} module in R (v. 2.4.0).

D. RESULTS

Variability in Acetaminophen-induced Liver Injury in Humans

To confirm a prior report of differential sensitivity to acetaminophen hepatotoxicity among healthy human volunteers[52], an independent cohort of 59 healthy subjects was enrolled in a double-blind, placebo-controlled study in which 49 received the maximum recommended therapeutic dose of acetaminophen (4 g/day for 7 days) and 10 subjects were randomly assigned to placebo. Elevations in ALT greater than 1.5-fold of individual baseline values were observed for 69% (34/49) of subjects receiving acetaminophen (Figure 2.1) and values exceeding 2-fold baseline were observed in 37% (18/49), confirming that some healthy subjects experience mild liver injury in response to therapeutic doses of acetaminophen. In each subject, a 1.5-fold cut-off was confirmed to represent significant ($P>0.05$) elevation in ALT from baseline by linear modeling. Interestingly, 31% (15/49) did not demonstrate ALT elevations greater than 1.5 fold baseline and showed no meaningful differences from the placebo-control group (N=10, $P=0.42$). ALT levels were at baseline levels in all subjects 14 days after

cessation of the treatment (Appendix 4). Elevations in serum ALT correlate well with other markers of liver injury (e.g., glutathione alpha-*S*-transferase) (Appendix 5).

Differences in Liver Injury in Mice Following Acetaminophen Exposure

To determine whether genetic factors influence acetaminophen-associated liver toxicity, a panel of 36 inbred mouse strains was selected to represent the genetic variation present within humans[64]. Liver toxicity was assessed at 4 and 24 hours after administration of an acute dose (300 mg/kg) of acetaminophen. Hepatic necrosis was histologically quantified 24 h after treatment and a dramatic interstrain variation in liver damage, exemplified by a characteristic centrilobular necrosis, was observed (Figure 2.2A, B). CAST/EiJ mice were most resistant as they sustained no liver necrosis or alterations in serum ALT, while B6C3F1/J mice, which are commonly used to evaluate chemical toxicity, were the most sensitive strain.

Serum ALT levels were measured at 4 and 24 hours post-dosing (Figure 2.2D, E). A Pearson correlation of 0.77 between serum ALT 24 h post-dosing with acetaminophen and extent of liver necrosis was noted, confirming that serum ALT is a good indirect marker for liver injury. However, comparison between ALT level at 4 and 24 hours post-dosing shows that it may be difficult to predict injury outcome from ALT measured at early time points following acetaminophen doses. These data suggest that

there are genetic factors that may independently affect the timing of acetaminophen-induced hepatocellular injury and ALT release.

It is well accepted that acetaminophen hepatotoxicity depends on metabolic activation, hepatic glutathione depletion and protein binding of NAPQI as initiating events. However, it is not known whether variability in glutathione levels and drug metabolism enzymes contribute to differential toxicity between individuals. Therefore, we quantified the ratio of reduced to oxidized glutathione in livers from mice sacrificed 4 h post-dosing, a time when acetaminophen-induced glutathione depletion is still robust[86]. There was no correlation between either reduced (Figure 2.2C) or total (data not shown) glutathione pool at 4 hours and liver necrosis at 24 hours post-dosing, suggesting that liver glutathione is not a sensitive biomarker for predicting injury outcome across individuals. Similarly, protein levels of cytochrome p450(CYP) 2E1, CYP1A2, catalase, and glutathione S-transferase (GST)pi in liver microsomes from mice sacrificed at 24 h did not correlate with acetaminophen-induced liver necrosis, or with serum ALT levels across individual strains (discussed in more detail in Chapter 4).

Inter-individual differences in pharmacokinetics of acetaminophen were found to be not correlated with liver injury in the previous study of acetaminophen hepatotoxicity among healthy human volunteers[52]. To investigate the inter-strain differences in metabolism of acetaminophen, we selected 5 strains (LP/J, C57BL/6J, DBA/2J, NZW/LacJ and C3H/HeJ) from our panel based on the differences in sensitivity to

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acetaminophen-induced liver necrosis (Figure 2.2A). The pharmacokinetics of the parent compound and its two major metabolites, sulfate and glucuronide conjugates, was assessed over a 6 h period following a bolus dose of 50 or 300 mg/kg (*i.g.*) using the area under the concentration (AUC) curve (Figure 2.3). After the 50 mg/kg dose, no differences between strains were observed (Figure 2.3A-C). After the 300 mg/kg dose, LP/J mice showed a significantly different profile than the other strains in exposure to acetaminophen and the glucuronide conjugate (Figure 2.3D-E), while no difference was observed for the sulfate conjugate. Despite the fact that metabolism of acetaminophen at high doses does vary between strains, this observation is insufficient to explain inter-individual differences in liver injury in mice, similar to that in humans, since susceptible strains have a much lower plasma exposure to acetaminophen than the resistant strains and therefore a theoretically lower exposure to NAPQI. Further analysis of pharmacokinetics in susceptible and resistant strains is presented in Chapter 4.

Representative mouse strains were selected from across the hepatic injury gradient to examine whether genetic variation also affects the dose-response. We classified each strain into one of three groups by the degree of necrosis observed 24 h following administration of 300 mg/kg acetaminophen. Representative non-responder (mean necrosis score less than 15%), mid-responder (mean necrosis score 15-50%), and high-responder (mean necrosis score > 50%) strains were tested at additional doses ranging from 30-1200 mg/kg (N=4). Markedly different dose-response curves in

response to acetaminophen were observed (Figure 2.2F). High-responder strains CBA/J, DBA/2J, and B6C3F1/J, and the mid-responder strain C57BL10/J have significant elevations in serum ALT at 24 h post-dosing with a 200 mg/kg dose. However, the high-responder strain C3H/HeJ and low-responder strain NOD/LtJ had no observable adverse response below 300 mg/kg. Of particular interest is strain CAST/EiJ in which comparably small elevations in ALT were observed only at 600, 900, or 1200 mg/kg.

Identification of Candidate Genes for Sensitivity to Acetaminophen-induced Liver Injury

To uncover polymorphisms associated with sensitivity to acetaminophen toxicity we performed haplotype-associated mapping utilizing a dense single nucleotide polymorphism (SNP) map[40]. Association analyses were performed with mouse serum ALT levels for 4 h (Figure 2.4A) and 24 h (Figure 2.4B) post-dosing. Because the genomic intervals with the greatest computed association with toxicity contained several genes (Table 2.1), we selected candidate genes that could be reasonably linked to the propagation of oxidative- or immune-mediated stress responses following acetaminophen exposure. We chose *Cd44, Cd59a, Ly86, Cat* and *Capn8* as likely candidate genes responsible for strain-specific liver injury.

A 300-800 bp region from each gene that contained either known non-synonymous coding SNPs or polymorphisms in intronic splice site regions was selected

for re-sequencing. Also included in the analysis was *Cyp2e1*, which is a primary enzyme known to metabolize acetaminophen to its reactive metabolite, NAPQI[87]. *Ly86, Cd44*, and *Cd59a* contain polymorphisms that, within the mouse diversity panel, correlate well with the degree of ALT release (Table 2.2; *P*<0.05). The *Capn8* gene, which was implicated in the 24 h ALT phenotype genome scan, was found to have a non-synonymous coding SNP that is highly correlative with 24 hour ALT measurements (*P*<0.05). The polymorphisms selected for genotyping in *Cat* or *Cyp2e1* were not correlative with markers of liver injury.

Mouse Genes Associated with Acetaminophen-induced Liver Injury Translate to Humans

To evaluate the human relevance of the susceptibility genes identified in mice, we tested if polymorphisms in orthologous genes correlate with inter-individual variability in acetaminophen toxicity in humans. We sequenced 300-650bp regions of *CD44, CD59, CAPN10* (human ortholog of mouse *Capn8*)*,* and *LY86* that included SNPs reported by the HapMap Project (http://www.hapmap.org) as having a minimal r^2 of 0.8 and a minor allele frequency greater than 0.05. Genomic DNA from two independent human cohorts were available for these experiments: a UNC cohort reported here and the Purdue Pharma cohort[52].

Within the UNC cohort, we observed an association between an individual's genotype at a *CD44* SNP (rs1467558) and the elevation in serum ALT reached during

the 14-day study (Figure 2.5A; *P*=0.02). The polymorphism is non-synonymous,

encoding an amino acid change from an isoleucine (C allele) to a threonine (T allele)

residue in the CD44 protein. In order to test whether this association is replicable, we

evaluated DNA from 76 subjects enrolled in the Purdue Pharma cohort. Because the

duration of acetaminophen administration was 14 days in the Purdue Pharma cohort (vs

7 days in the UNC cohort), data analysis was limited to the first seven days of treatment.

Within the Purdue Pharma cohort, a C/T genotype at the same *CD44* SNP (rs1467558)

was also found to be associated with ALT elevations during treatment (Figure 2.5B;

P=0.01). When the two cohorts were combined, the association was more significant

(Figure 2.5C; *P*=0.002). To further assess the functional relevance of this finding to

acetaminophen-induced liver injury in mice and humans, we performed experiments in

Cd44-null mice and performed *in silico* prediction of the effect of the amino acid

substitution resulting from the polymorphism at *CD44* SNP rs1467558. Indeed, *Cd44*-

null mice exhibit significantly greater liver injury 24 h following administration of

acetaminophen (300 mg/kg) as compared to the wild type (C57BL/6J) counterparts

(Figure 2.6). Furthermore, the structural ramification of the change from isoleucine (C

allele) to threonine (T allele) in the CD44 protein due to SNP rs1467558 was predicted

in silico to be possibly damaging to the function of the protein due to the potential

creation of a cavity within a buried site with a PolyPhen PSIC score difference between

the variant proteins of 1.711.

A polymorphism within *CAPN10* (rs3749166) displayed a trend across both cohorts in which individuals having the G/A allele tended to be more sensitive to acetaminophen-induced ALT elevations in the first seven days of treatment (Figure 2.5D-F). While the trend remained consistent across sample populations, the data was only marginally significant when analyzed in the combined cohorts (*P*=0.045). It is interesting to note that while rs3749166 is a synonymous coding SNP, it is a tag SNP for rs2975766, a non-synonymous polymorphism that alters coding from isoleucine to valine.

There was no correlation between increased serum ALT and genotyped polymorphisms within the *CD59* (rs10538602) or *LY86* (rs5874047) genes in the data collected. There was also no statistical difference between sensitivity to acetaminophen and genotype when all pairs of gene-gene interactions were examined (data not shown).

E. DISCUSSION

One of the major reasons that efficacious drugs fail to advance through late stages of development, or are removed or restricted after entering the marketplace, are rare adverse health events that were not predicted using current preclinical testing paradigms[68]. Consequently, being able to identify which drugs cause, and more importantly which individuals are likely to develop, adverse reactions is a major

challenge preventing informed deployment of new medicines. The novel experimental approach we describe here, using acetaminophen as a model compound, bypasses the limitations of humans-only pharmacogenetics studies by showing that a population of mouse strains can be used to predict genetic biomarkers of toxicity sensitivity.

A traditional genome-wide pharmacogenetic investigation[88] into the genetic factors linked to the liver toxicity of acetaminophen would require a much larger number of individuals due to greatly reduced power associated with P-value correction in whole-genome SNP analyses. Conversely, a so called "candidate gene" analysis[89] may be equally challenging due to a complexity of the mechanism of action of acetaminophen[54]. Significantly, well characterized genes known to be essential for acetaminophen toxicity did not correlate with liver injury in the panel of mouse strains. Rather, the candidate susceptibility genes identified through genetic studies in the mouse translated to two independent human cohorts despite small numbers of individuals available.

It is noteworthy that the top candidate genes suggested by the analysis of the inbred mouse strains are related to the immune response, and not to metabolism and detoxification of acetaminophen. The traditional view on the mechanisms of toxicity, the approach widely utilized to predict individual responses to xenobiotics, would imply that metabolism of acetaminophen to the reactive electrophile NAPQI and/or detoxification of the latter by glutathione conjugation should explain, at least to a considerable degree, the variability in responses. However, no apparent correlation between levels of major

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metabolizing enzymes, glutathione, or acetaminophen plasma exposure in select strains and liver injury was observed in the mouse population. Similarly, in several cytokine knockout mouse models of acetaminophen toxicity, the sensitivity to liver necrosis due to acetaminophen was largely independent of covalent binding of NAPQI to proteins or glutathione depletion[54]. Furthermore, we found no correlation with sensitivity for polymorphisms in the genes encoding catalase or cytochrome P450 2E1, implying that variation at these key mediators of acetaminophen toxicity cannot fully explain differential susceptibility to acetaminophen. This conclusion does not refute the molecular mechanism of APAP toxicity via bioactivation by CYP2E1. In contrast, our data form a basis by which we show that the *end outcome* of the toxicity response is not directly correlative with inter-individual differences in the basic metabolism of acetaminophen. This indicates that other cellular processes leading to tissue injury, in addition to metabolism and pathways involved in cell damage, are involved in determining the extent of liver necrosis observed following treatment with acetaminophen. This also raises a critical distinction between genes (enzymes/proteins) that are essential mediators of toxicity but which may not functionally vary (*e.g.* CYP2E1) and those, whose activity or function may vary considerably among individuals and determine susceptibility to toxicity (*i.e.* CD44, see below).

While events downstream of the consumption of hepatic intracellular glutathione are not as well described as acetaminophen metabolism, these downstream events

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have been shown to be a major mediator of the toxicity response. Indeed, the presence

of inflammatory mediators released from non-parenchymal cells in the liver, including

interleukin-(IL)6[90], IL-10[91], interferon-γ[92], and tumor necrosis factor-α[93], have been

shown to affect liver sensitivity to acetaminophen. Furthermore, neutrophil-mediated

necrosis[60] and Kupffer cell recruitment[94] have also been implicated as important factors

in progression of liver injury; however, their precise role and timing of involvement are

debated[62, 95].

Our data supports the notion that variation in immune response may be the most

critical of the complex events that determine susceptibility to acetaminophen toxicity

since a number of candidates from this pathway were significantly associated with

strain-specific injury in response to acetaminophen. Within the mouse diversity panel,

ALT release at four hours was shown to be affected by polymorphisms in lymphocyte

antigen 86 (*Ly86,* also known as *MD-1*), CD44 antigen (*Cd44*), and CD59a antigen

(*Cd59a),* which are involved in B-cell responsiveness to lipopolysaccharide, lymphocyte

adhesion and activation, and regulation of complement deposition, respectively. Subtle,

transient alterations in immunogenic signaling during acetaminophen toxicity may also

play a role in the development of idiosyncratic toxicities in an individual, however more

data is needed to fully characterize this relationship.

Capn8, a gene identified by association mapping of the 24 hour ALT phenotype,

was the only non-immune gene found to be associated with sensitivity to

acetaminophen (an exonic A to G base change). This observation is intriguing given that calpain released from necrotic hepatocytes has been associated with the progression of acetaminophen-induced liver injury[96]. In addition, calpastatin, a specific inhibitor of calpain, was recently shown to play a role in attenuating liver injury and increasing survival of mice following an acute dose[97].

The ability of the panel of mouse strains to predict sensitivity to acetaminophen-induced liver injury in humans was supported by sequencing of the orthologous genes positively associated with liver injury in mice. Consistent with the data in the mouse population, we found *CD44* to be a marker of sensitivity in two independent human cohorts. The genotypes at *CD44* allowed partitioning of subjects based upon susceptibility to acetaminophen-induced hepatic toxicity and implicate variation in immunogenic cell surface antigens as potential mediators of acetaminophen sensitivity. It is noteworthy that heterozygous (C/T) individuals are more susceptible, since (i) *in silico* prediction of the effect of this non-synonymous coding SNP suggests a disruption in the protein function and (ii) Cd44-null mice are more susceptible to liver necrosis due to acetaminophen. These data are intriguing given that *Cd44*-deficient mice exhibit greater liver injury due to another classic hepatotoxicant, carbon tetrachloride[98]. Interestingly, inflammatory response to carbon tetrachloride was temporally shifted in *Cd44*-deficient mice compared to wild-type (C57BL/6 mice), an effect that may be mediated by the temporal differences in liver NF-κB activity. Therefore, it is possible that

variations in *CD44* may significantly affect liver necrosis through effects on leukocyte signaling via cytokine modulation. However, owing to the many physiologic and pathologic roles of CD44 isoforms *in vivo*[99], including cell-cell matrix interaction, lymphocyte extravasation, wound healing, scar formation, cell migration, and the binding and presentation of growth factors, the precise mechanistic role of this gene in conferring sensitivity to acetaminophen-induced ALT elevations remains to be determined.

F. CONCLUSIONS

Collectively, our results indicate that the use of an inbred mouse strain panel is a valuable tool for evaluating drug safety and for the development of biomarkers to pre-screen individuals prior to therapeutic drug treatment with potential toxicities. The identification of the genes associated with differential susceptibility to toxicity in a pre-clinical phase, exemplified by the finding that CD44 may be involved in modulation of susceptibility to acetaminophen hepatotoxicity, has potential to focus pharmacogenetics research, overcome the challenge of small human cohorts, and to shorten the validation period. The data acquired with this model could therefore be influential in the analysis of individual risk to pharmaceutical agents and may facilitate both drug development and human safety endeavors. One of the limitations of this approach, however, lies in the uncertainties of whether the associations between SNPs and modest increases in ALT

observed with "therapeutic" doses would also predict individuals susceptible to more severe toxicity seen in overdose situations. Additional research into the mechanisms of predisposition to minor forms of liver injury and those which lead to more severe organ damage is needed.

Table 2.1

Genomic regions identified by haplotype-associated mapping in inbred mouse strains

Phenotype	Peak	Genome position (Mb)	Genes in region
4 Hr ALT	1	Chr 2: 102.08-106.96	*Trim44, E430002G05Rik, Slc1a2,* **Cd44**, *Pdhx, Apip, Ehf, BC016548, Elf5,* **Cat**, *Abtb2, Nat10, Gpiap1, Lmo2, 4931422A03Rik, Fbxo3, Cd59b,* **Cd59a**, *A930018P22Rik, D430041D05Rik, Hipk3, Cstf3, Tcp11l1, AV216087, Qser1, Prrg4, Ccdc73, Ga17, Wt1, 0610012H03Rik, Rcn1, Pax6, Elp4, Immp1l, Zcsl3, 4732421G10Rik, Mpped2, 2700007P21Rik, Fshb*
	2	Chr 3: 126.439–26.844	*Ank2*
	3	Chr 4: 141.531–43.578	*Prdm2, Pdpn, Lrrc38, Pramel1, 4732496O08Rik, Oog4, BC080695, Pramel5, Pramel4, Oog3*
	4	Chr 6: 123.795–24.766	*V2r1b, Cd163, Pex5, Clstn3, C1rl, C1r, Oact5, Emg1, Phb2, Ptpn6, Grcc10, Atn1*
	5	Chr 13: 36.862–37.022	**Ly86**
	6	Chr 17: 5.598–5.655	*Zdhhc14*
24 Hr ALT	7	Chr 1: 182.602–82.719	**Capn8**
		Chr 1: 189.550–89.735	*Prox1*
	8	Chr 2: 127.489–27.580	*Bub1, 1500011K16Rik*
	9	Chr 4: 124.084–24.395	*Utp11l, Fhl3, Sf3a3, Inpp5b, Mtf1, Yrdc, Gm50, Epha10, Cdca8, 9930104L06Rik*
	10	Chr 5: 97.392–97.681	*Prdm8, Fgf5, 1700007G11Rik*
	11	Chr 7: 86.492–86.594	No known genes

Genes highlighted in bold were selected for sequence analysis.

Table 2.2

Sequence analysis of polymorphisms within candidate mouse regions

Gene	Genomic location	Genotype	No. of Strains	4hr ALT (Mean ± SE)	4hr ALT P value	24hr ALT (Mean ± SE)	24hr ALT P value
Cyp2E1	135176451	T	2	1304 ± 619	0.4733	8205 ± 3128	0.6972
		C	22	1822 ± 323		6355 ± 756	
Catalase	103162120	T	3	149 ± 52	0.2527	5381 ± 1430	0.2842
		C	3	1239 ± 409		1683 ± 928	
		A	9	2563 ± 693		7578 ± 1401	
		deletion	11	1704 ± 466		5718 ± 1057	
	103162021	C	1	3271 ± 1373	0.2527	8463 ± 3202	0.7456
		T	2	50 ± 7		5272 ± 1873	
		deletion	23	1817 ± 351		5790 ± 759	
Lymphocyte antigen 86	**36798778**	G	7	785 ± 245	**0.0024**	4455 ± 1126	0.2842
		A	21	2465 ± 388		6724 ± 823	
	36798990	C	10	780 ± 192	**0.0012**	5062 ± 954	0.3201
		A	18	2674 ± 430		6722 ± 892	
CD44 antigen	**102693730**	C	6	4731 ± 1110	**0.0180**	9283 ± 1773	0.2508
		G	22	1309 ± 234		5232 ± 734	
	102693564	G	14	3058 ± 593	**0.0176**	7535 ± 1121	0.2842
		A	14	1140 ± 270		4991 ± 888	
CD59a antigen	**103896673**	T	12	1342 ± 399	**0.0488**	5287 ± 868	0.3201
		A	16	2716 ± 451		6964 ± 1022	
	103896938	T	12	1342 ± 399	**0.0488**	5287 ± 868	0.3201
		C	16	2716 ± 451		6964 ± 1022	
Calpain 8	182615475	T	3	3161 ± 952	0.2853	7793 ± 2660	0.6877
		C	25	1953 ± 337		5963 ± 713	
	182615494	C	2	1302 ± 652	0.2853	5509 ± 1485	0.7221

	T	26	2171 ± 339		6255 ± 756	
__182615696__	A	2	852 ± 550	0.0963	2095 ± 461	__7.39E-05__
	G	26	2204 ± 339		6458 ± 744	

P values < 0.05 are in bold. Underlined genomic locations indicate that the polymorphism causes a non-synonymous amino acid change in the protein.

Figure 2.1

Maximum serum ALT fold change measured in human volunteers taking daily oral doses of acetaminophen

The peak ALT fold change over baseline reached over the course of treatment by each subject in the UNC cohort is shown. Subjects were considered responders (white bars, N=34) if peak serum ALT reached greater than 1.5-fold (line) higher than the subject's baseline value. Black bars represent subjects who were non-responders (*i.e.* with a peak ALT fold change less than 1.5).
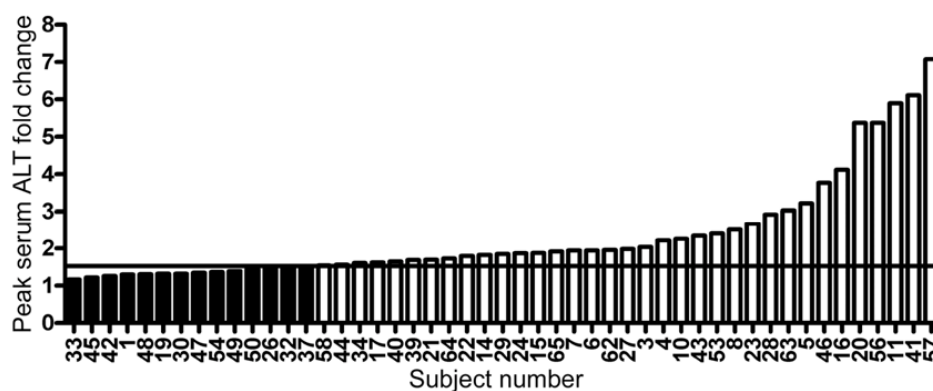
Figure 2.2

Toxicity responses to acetaminophen in a panel of mouse strains

(A) Representative photomicrographs (100x) of the hematoxylin-eosin stained sections of left liver lobe of mice 24 hours after dosing with acetaminophen (300 mg/kg). (B) Liver necrosis score (mean±S.E., n=3-4/strain) in mice treated with acetaminophen (300 mg/kg) for 24 h. (C) Serum ALT levels (mean±S.E.) in acetaminophen-treated mice sacrificed 24 h after dosing. (D) Serum ALT levels (mean±S.E.) in acetaminophen-treated mice sacrificed 4 h post-dosing. (E) Liver reduced glutathione (ratio between acetaminophen- and vehicle-treated animals in each strain, mean ± S.E.) 4 h post-dosing. Symbol (❖) indicates strains with no data. (F) Dose-response to acetaminophen-induced liver injury as measured by ALT release (n=4/strain, mean±S.E.) at 24 h after treatment.

a 24hr Liver Histopathology

Cast/EiJ   SM/J   C57BL/6J   DBA/2J   B6C3F1/J

b 24hr Necrosis Score

c APAP/Control Red. GSH

d 24hr ALT, U/L, x 10^3

e 4hr ALT, U/L, x 10^3

CAST/EiJ, CZECHII/EiJ, NZO/LtJ, C57L/J, NOD/LtJ, LP/J, PLJ, WSB/EiJ, BTBR t+ tf/J, SWR/J, A/J, SM/J, PWD/PhJ, NON/LtJ, MA/MyJ, RIIIS/J, SJL/J, C57BL/6J, C57BLKS/J, MSM/Ms, PERA/EiJ, SEA/GnJ, AKR/J, FVB/J, C57BR/CdJ, 129S1/SvlmJ, BALB/cByJ, DBA/2J, JF1/Ms, PJ, C57BL/10J, BUB/BnJ, KK/HlJ, NZW/LacJ, C3H/HeJ, CBA/J, B6C3F1/J

f

Legend:
- CAST/EiJ
- AKR/J
- B6C3F1/J
- C3H/HeJ
- C57BL/10J
- CBA/J
- DBA/2J
- LP/J
- NOD/LtJ

24hr Serum ALT, U/L

Acetaminophen dose, mg/kg

Figure 2.3

Plasma AUC of acetaminophen metabolites

Plasma AUC of acetaminophen (mean±S.E.) as well as the glucuronide and sulfate conjugates measured across strains for 6 h post-dosing with (A-C) 50 mg/kg (*i.g.*) or (D-F) 300 mg/kg (*i.g.*) following an overnight fast. Asterisk (*) indicates significant differences between strains by the Tukey post-hoc test (*P*<0.05).
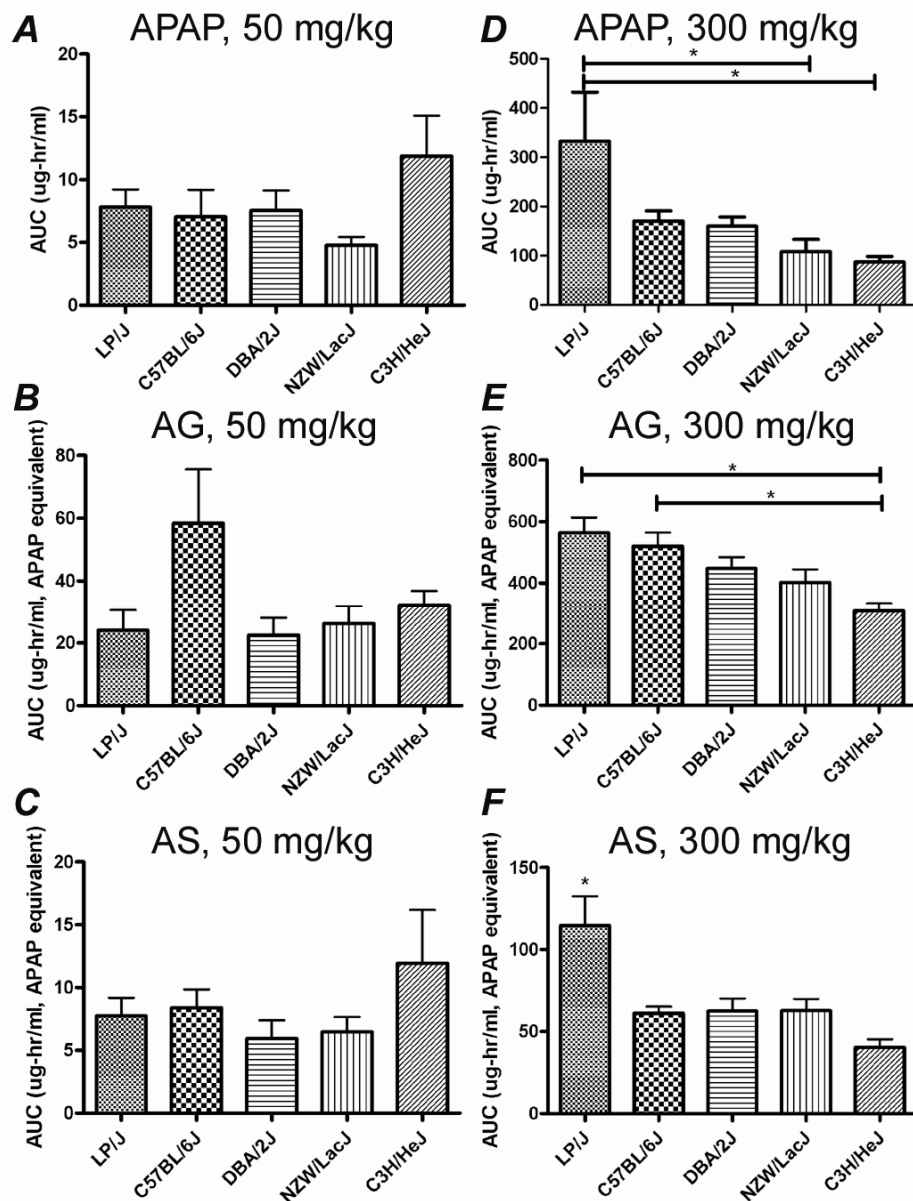
Figure 2.4

Haplotype association mapping of acetaminophen-induced liver injury in the mouse

Serum ALT at 4 (A) and 24 (B) hours after acetaminophen (300 mg/kg) treatment was used to identify genomic intervals significantly associated with liver injury. Peaks (numbered, see Table 2.1) indicate a significant log$P$ association score at each 3-SNP marker window. Marker colors indicate chromosome number across the mouse genome.
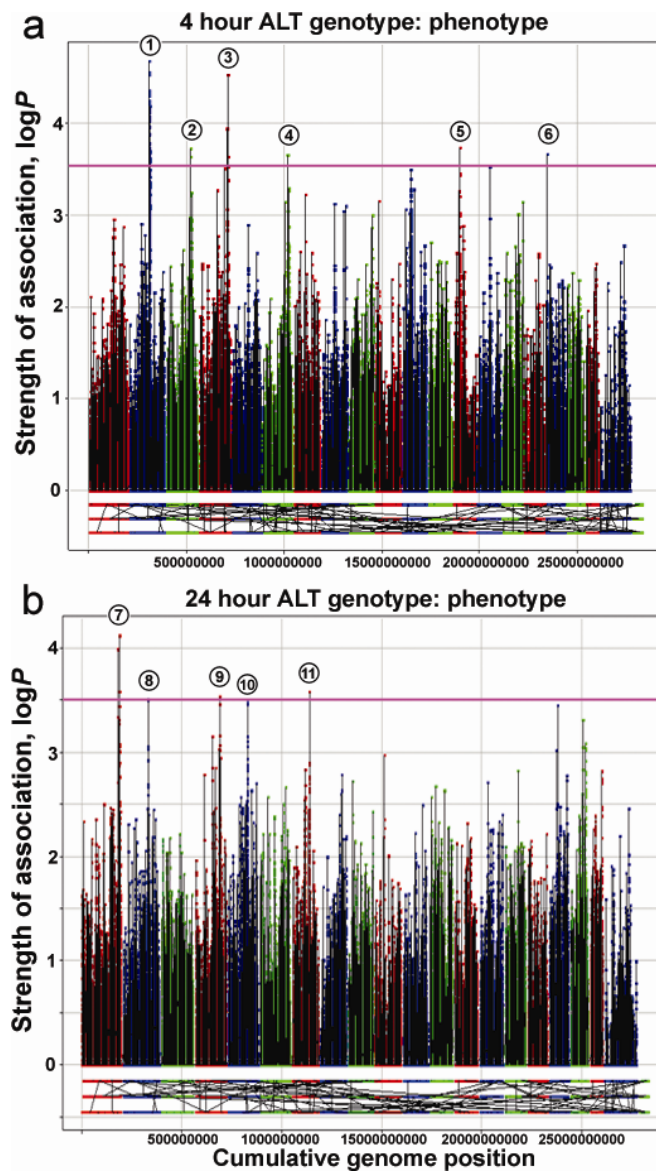
Figure 2.5

ALT elevations in human volunteers delineated by genetic variation in *CD44* and *CAPN10*

Polymorphisms in *CD44* (A-C) and *CAPN10* (D-F) associated with susceptibility to acetaminophen-induced liver injury in humans. Data from UNC (A and D), Purdue Pharma (B and C) and a combined cohort (C and F) are shown. Average mean (± S.E.) serum ALT per genotype is plotted for each matching study day.
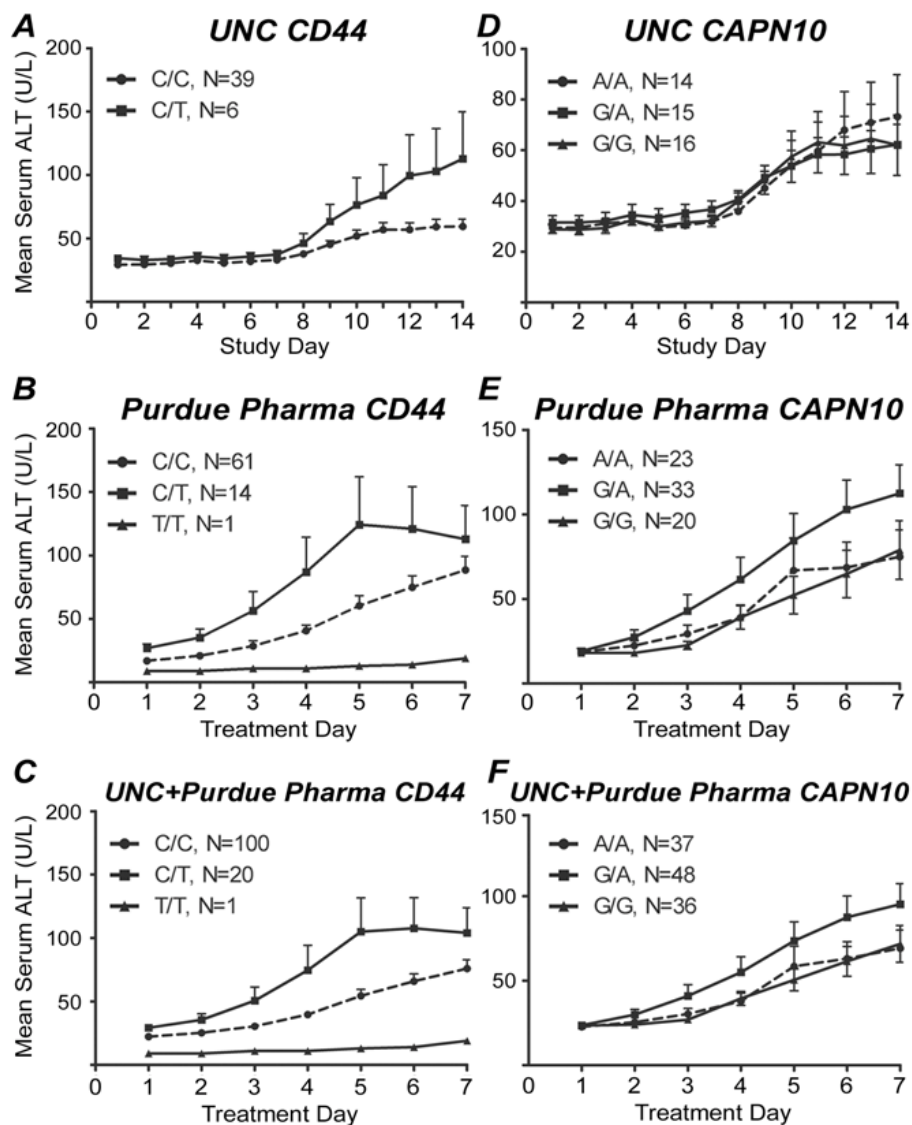
Figure 2.6

Acetaminophen-induced liver injury in *Cd44* gene knockout and wild type mice

Differential susceptibility of CD44 knockout (KO) and wild-type (WT) mice to liver injury following an acute dose of APAP (300 mg/kg, *i.g.*) as measured by percent liver necrosis (mean±S.E.). Asterisk (*) indicates a significant difference between groups (P<0.05).
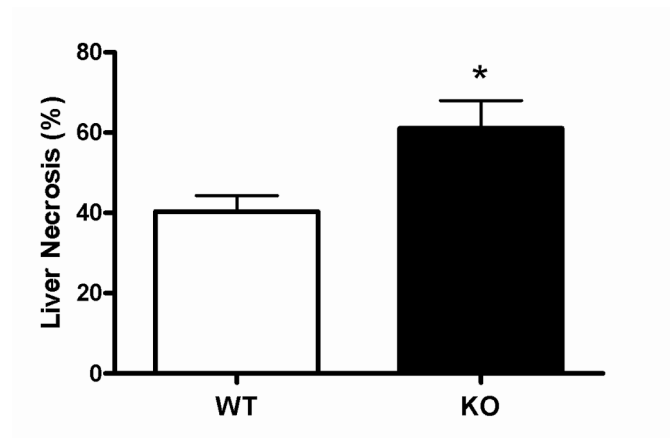
Chapter 3

POPULATION-BASED DISCOVERY OF TOXICOGENOMICS BIOMARKERS FOR
HEPATOTOXICITY USING THE MOUSE MODEL OF THE HUMAN POPULATION

A. ABSTRACT

Toxicogenomic studies are increasingly used to uncover potential biomarkers of adverse health events, enrich chemical risk assessment, and to facilitate proper identification and treatment of persons susceptible to toxicity. Current approaches to biomarker discovery through gene expression profiling usually utilize a single or few strains of rodents, limiting the ability to detect robust biomarkers that may represent the wide range of toxicity responses typically observed in genetically heterogeneous human populations. To enhance the utility of animal models to detect toxicity biomarkers for genetically diverse populations, we used a laboratory mouse strain diversity panel. Specifically, mice from 36 inbred strains derived from *Mus musculus musculus*, *M.m. castaneous*, and *M.m. domesticus* origins were treated with a model hepatotoxicant, acetaminophen (300 mg/kg, *i.g.*). Gene expression profiling was performed on liver tissue collected at 24 hours after the dose. We identified 26 population-wide biomarkers of response to acetaminophen hepatotoxicity in which the changes in gene expression were significant across treatment and liver necrosis score, but not significant for individual mouse strains. Importantly, these genes point to a sub-set of the intracellular signaling involved in acetaminophen-induced hepatocyte death, such as *oncostatin M receptor* and *MLX interacting protein-like*. These data demonstrate that a multi-strain approach may provide a more robust method for understanding genotype-independent toxicity responses and identify novel targets of therapeutic intervention.

B. INTRODUCTION

Biological monitoring to assess potential toxicity of chemical and pharmaceutical compounds relies heavily on the availability of sensitive, specific and widely-applicable biomarkers of toxic effects[100]. Toxicogenomics is widely used at all stages of chemical risk assessment and it is thought that gene expression changes may be utilized as biomarkers of adverse effects [101]. Current approaches often attempt to classify compounds with the goals of predicting adverse responses to specific chemical classes[48], understanding the underlying biological mechanism of toxicity[102], or identifying key nodes in the toxicity pathway that may serve as biomarkers[103]. Extensive proprietary[104-106] and public[107, 108] databases containing gene expression profiles and pathological endpoints derived from rodent and human tissues exposed to a variety of chemicals have been developed, thereby allowing the scientific community to mine the data for toxicity biomarkers of interest.

Many biomarkers of toxicity may be surrogate measures for the genetics of an individual, which can play a major role in determining the threshold of toxicity of a given compound[69]. Compelling research has led to the identification of gene variants that correlate with drug toxicity[109] and recent pharmacogenomic research efforts have made significant advances in connecting variability in responses to drug efficacy and/or toxicity to genetic polymorphisms[33]. While major research efforts are seeking genetic and genomic markers that could identify individuals susceptible to toxicity, less attention is

given to the fact that inter-individual variability in responses and genetic control of gene expression[110, 111] may present a challenge for finding robust population-wide expression biomarkers of effect. Indeed, while toxicogenomics has been used widely for the study of toxicity biomarkers across compounds and across species, its usefulness in determining biomarkers that are relatable to a genetically diverse human population is limited by a lack of intra-species comparisons.

To address the need for a biomarker identification strategy that is independent of population heterogeneity, we utilized a mouse Laboratory Strain Diversity Panel[80]. The use of a genetically-defined panel of mice has advantages over classical toxicology testing strategies that utilize a single inbred or outbred strain because it takes advantage of the vast genetic diversity that is available among inbred mouse lines[74, 112]. We hypothesized that toxicity responses across a panel of strains will produce a range of effects similar to those expected to occur in human populations, and that this phenotypic diversity can be used to identify population-dependent and –independent mRNA transcript biomarkers of response. Equally important to the model design is the genetic homogeneity that exists within a strain, enabling repeated testing from a specific genotype. To test our hypothesis, we selected the classical hepatotoxicant acetaminophen. We observed a dramatic gradient of acute hepatotoxicity across strains and the analysis of liver gene expression data revealed 26 genes that correlated with liver necrosis outcome and were not affected by genetic differences between individual

strains. Thus, these genes, the majority of which are tightly linked in a cell death and proliferation network, can serve as robust biomarkers for predicting responses across a genetically heterogeneous population.

C. METHODS

Mice

Male mice (aged 7-9 weeks) were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in polycarbonate cages on Sani-Chips irradiated hardwood bedding (P.J. Murphy Forest Products Corp., Montville, NJ). Animals were fed NTP-2000 wafer diet (Zeigler Brothers, Inc., Gardners, PA) and water *ad libitum*, and maintained on a 12 h light-dark cycle. Mice utilized in this study comprise 36 inbred strains that are priority strains for the Mouse Phenome Project[80]: 129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BTBR T+ tf/J, BUB/BnJ, C3H/HeJ, C57BL/10J, C57BL/6J, C57BLKS/J, C57BR/CdJ, C57L/J, CAST/EiJ, CBA/J, CZECHII/EiJ, DBA/2J, FVB/NJ, JF1/Ms, KK/HlJ, LP/J, MA/MyJ, MSM/Ms, NOD/ShiLtJ (formerly NOD/LtJ), NON/LtJ, NZO/H1LtJ, NZW/LacJ, P/J, PERA/EiJ, PL/J, PWD/PhJ, RIIIS/J, SEA/GnJ, SJL/J, SM/J, SWR/J, and WSB/EiJ. F1 hybrid mice, B6C3F1/J, were also used for phenotypic measurements. These studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

Acetaminophen administration and sample collection from mice

Mice were singly housed and fasted 18 h prior to intra-gastric dosing with acetaminophen (99% pure, Sigma-Aldrich, St. Louis, MO; N=3-4 per strain) or vehicle (0.5% methyl 2-hydroxyethyl cellulose, Sigma-Aldrich; N=2 per strain, except for strains PERA/EiJ, SWR/J, and CZECHII/EiJ (N=3), as well as strains AKR/J, and CAST/EiJ (N=1, *i.e.* sufficient tissue was not available). The dose of 300 mg/kg was delivered in 10 ml/kg of the vehicle. Dosing was performed at the same time of day (9 am) throughout the study as diurnal effects have been shown to affect gene expression in rodent studies[81]. Feed was returned 3 h after dosing; animals were necropsied 24 h after treatment (Nembutal 100 mg/kg *i.p.*, Abott Laboratories, Chicago, IL). Livers were quickly excised following ex-sanguination and sections of the left lateral lobe were placed in 10% phosphate buffered formalin for immunohistochemical analyses. Remaining liver from the left lobe was snap-frozen in liquid nitrogen and stored at - 80ºC for RNA extraction.

Liver histopathology

Paraffin-embedded liver tissue was cut to 5 µm sections in duplicate and stained with hematoxylin and eosin (H&E). Liver injury in the left liver lobe was blindly scored by A.H. and confirmed by a certified veterinary pathologist. Necrosis was quantified by unbiased stereology using a point counting technique[83]. Briefly, a grid with 100 evenly spaced points was overlaid on printed images of liver sections taken at 100X

magnification. The total number of points lying in an area of necrosis was divided by the total number of points lying completely within the entire tissue section to determine a percent necrosis score (0-100%).

RNA isolation

To eliminate variability in transcript expression that might arise between liver lobes, the left liver lobe was selected for the remainder of the data analysis and gene expression profiling. RNA was extracted from the 30 mg of tissue derived from the left lobe of sample livers using the Qiagen RNeasy kit (Qiagen, Valencia, CA). RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality was verified using the Agilent Bio-Analyzer (Agilent Technologies, Palo Alto, CA).

Microarray hybridizations

In this study, all RNA samples were hybridized to arrays individually; none were pooled. RNA amplifications and labeling were performed using Low RNA Input Linear Amplification kits (Agilent Technologies). For hybridization, 750 ng of total RNA from each mouse liver was amplified and labeled with fluorescent dye (Cy5). In parallel, 750 ng of a common reference RNA (Icoria Inc., RTP, NC) was labeled with the fluorescent dye, Cy3, in order to standardize analysis of global gene expression between mouse strains[4]. Labeled cRNA was then processed and hybridized to Agilent Mouse Toxicology Arrays (catalog# 4121A, about 22,000 features) according to the

manufacturer's protocol. Following hybridization, arrays were washed using a custom protocol developed by Icoria, Inc. Briefly, array gaskets were removed under immersion in Wash Solution 1 (6X SSPE, 0.005% N-Lauroylsarcosine). Arrays were washed with Wash Solution 1 and incubated for one minute with gentle agitation on a magnetic stir plate. A second incubation was performed in Wash Solution 2 (0.06X SSPE, 0.005% N-Lauroylsarcosine).

Data analysis of significantly changed transcripts

Raw microarray intensity values were obtained from Agilent Feature Extraction software (v8.5) and archived in the UNC Microarray Database (http://genome.unc.edu). Raw data is available to the public through this database. The $\log_2$ ratio of Cy5/Cy3 intensity was normalized using LOWESS smoothing to eliminate intensity bias of features. Transcripts with fewer than 70% good data across samples were excluded from the analysis, reducing the probe list to 15,509 transcript probes. Intensity ratios were transformed to eliminate hybridization batch effects using the Batch Normalization feature in Partek Genomics Suite (Partek Inc., St. Louis, MO). Analysis of significant transcripts was performed using an ANCOVA model in Partek in which the main effects were mouse strain, treatment, the interaction of mouse strain and treatment, and the sample necrosis score. Transcripts were called significantly different if the p-value was less than a threshold determined by a step-down false discovery rate[113] (FDR, $\alpha=0.01$)

to correct for multiple comparisons across array features. Heat maps were generated using hierarchical agglomerative clustering.

Functional analysis of significant genes

Onto-Tools Onto-Express (http://vortex.cs.wayne.edu/)[114] was used to generate functional profiles of the significant transcripts identified by the ANCOVA model for each factor. Significance values were calculated based upon a hypergeometric distribution. A *P* value cutoff of *P*<0.05 was selected as the cutoff for significance of expanded gene ontology categories. The gene network of the 26 response biomarkers was prepared by determining connecting nodes, interactions, and cellular compartments with Ingenuity Pathway Analysis software v. 5.5 (Ingenuity Systems, Redwood City, CA).

D. RESULTS

Histopathology of liver toxicity across inbred mouse strains

At 24 h after dosing with 300 mg/kg of acetaminophen (*i.g.*) we observed centrilobular necrosis in the liver consistent with that previously reported for acute doses of acetaminophen[115, 116]. Necrosis was accompanied by inflammatory infiltration into the hepatic parenchyma and, in varying degrees, hemorrhage was also present. Quantitative liver necrosis scores reflective of the proportion of the affected area were obtained from the left liver lobe[117] and demonstrated a wide range of toxicity across the panel of inbred mouse lines (Figure 3.1). The rank order of sensitivity to acetaminophen-

induced liver injury across strains shows that the majority of tested strains (30/36) sustained less than 40% liver necrosis, while 6 strains sustained liver necrosis of between 40-100%.

Determination of Gene Transcripts Associated with Strain, Treatment and Liver Necrosis

Gene expression values were collected on individual animals in this study (vehicle and acetaminophen-treated mice) and used for principal components analysis to visually examine the patterns in global mRNA transcript differences (Figure 3.2). The unsupervised analysis displayed separation of the samples by both treatment and by the amount of liver necrosis sustained in the animal indicating that gene signatures may be determined that are correlative with liver toxicity due to acetaminophen.

To determine those transcripts in which expression was significantly differentiated among the experimental factors, an ANCOVA (analysis of covariance) model was used. Covariate factors for each individual mouse included the strain (genotype), treatment (vehicle or acetaminophen), the interaction between strain and treatment because of anticipated genotype-specific effects on acetaminophen metabolism and transport, and the liver necrosis score. The number of transcripts significantly changed among each experimental factor is depicted in a Venn diagram (Figure 3.3A) and excludes those genes with a significant strain-by-treatment interaction.

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Interestingly, the majority of genes (1524) found to be significantly different between samples in the ANCOVA analysis were attributed to the strain effect, not acetaminophen treatment, or the degree of liver necrosis. This strain-specific gene set best represents those genes that differ in basal levels among the panel of inbred mouse strains and whose expression is likely to be affected by genetic polymorphisms[111].

Next, Gene Ontology (GO) analysis was performed in order to determine biological pathways most affected by the experimental factors of strain, treatment, or liver injury, alone and in combination (Table 3.1). There were few GO categories that were identified as significant for necrosis alone, and there were no broad molecular or biological processes affected by treatment alone. Categories enriched for genes significant by both strain and necrosis, but not by treatment, included protein binding, ATP binding, and structural elements of tissue. This gene set represents those genes that have a significant association with the liver necrosis value and that are dependent upon an individual's genotype, but independent of whether the animal received acetaminophen or vehicle.

The functional profile for those genes that were significant for all three factors (strain, treatment, and necrosis) was found to be associated with microtubule binding. This gene set represents those genes that could yield important information on the mechanism of acetaminophen toxicity, but would make a poor biomarker because basal levels are affected by individual genotype. It should be noted that the two significant GO

categories for this set are both driven by the inclusion of the gene *Mapre1* (microtubule-associated protein, RP/EB family, member 1).

Population-Based Gene Expression Biomarkers of Response

There were 26 transcripts whose expression was affected significantly by both treatment and by the toxicity outcome (*i.e.,* liver necrosis), but not the subject's genotype (Table 3.2). We reason that these genes could serve as population-based biomarkers of response. A heat map was generated to visualize gene expression changes in these biomarker transcripts across individuals (Figure 3.3B). A clear gradient of expression changes can be observed for each of these genes depending on the amount of necrosis sustained by an individual mouse. Expression of 17 of these transcripts increased, while nine genes decreased as liver necrosis increased in acetaminophen-treated mice (Table 3.2). Functional analysis of these genes revealed significant over-representation of four molecular functions, including hematopoietin/interferon-class (D200-domain) cytokine receptor activity, proteasome activator activity, cyclin binding, and MAP kinase activity (Table 3.1).

In order to determine whether molecular interactions exist among the population-based transcript biomarkers, a pathway map was constructed using Ingenuity Pathway Analysis. This analysis revealed that 16 of the 26 population-based response biomarkers are closely linked in a cell death and proliferation network centered on cell

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cycle regulating genes *Trp53*, *Myc*, *Jun*, and *Cdkn1a* (*p21*) (Figure 3.4). Closely associated with this network were the cytokine-responsive genes interleukin 6 signal transducer (*Il6st*) and oncostatin M receptor (*Osmr*) (Figure 3.3C-D), as well as the glucose-responsive transcription factor MLX interacting protein like (*Mlxipl*) and cell cycle gene CDC14 cell division cycle 14 homolog B (*Cdc14b*) (Figure 3.3E-F).

E. DISCUSSION

Decades of mechanistic investigations into the liver toxicity of acetaminophen have concluded that: (i) metabolic activation to the reactive metabolite N-acetyl-*p*-benzoquinone imine and its binding to cellular proteins is an essential initiating event for the toxicity; (ii) intracellular events involved in cell death such as mitochondrial dysfunction and formation of reactive oxygen and nitrogen species propagate the injury; and (iii) inflammatory response to cell death in the liver may exacerbate the damage[54, 77]. Thus, the fact that our study not only identified 26 biomarker genes in which expression across strains was associated with the level of liver necrosis, but also showed that 16 of these genes are involved in cell death pathways and form a closely linked molecular network, confirms a central role for intracellular cell signaling in acetaminophen-induced liver toxicity.

Not only are cell death-related genes mechanistic biomarkers of effect across genetically diverse individuals as identified in our work, they also have been shown to be

consistently affected and significantly correlated with the acetaminophen-induced liver toxicity phenotype in a multi-center toxicogenomic study[118]. The study, conducted at seven different laboratories around the U.S., used only one inbred strain, C57BL/6J; however, it showed that *Myc* is induced by acetaminophen and that a MYC–centered cell death pathway is the most significant network of proteins associated with liver injury in the mouse at 6, 12 and 24 hours after treatment with a dose identical to that used in our work. Furthermore, expression of Cdk inhibitor p21 (*Cdkn1a*), a central gene in the biomarker gene network, has been shown previously to be required for liver necrosis in rodents[119]. In addition, decreased levels of *Cdc14b* are consistent with increased activation of *Trp53*[119], which may be a compensatory mechanism to signal for an increase in cellular repair following acetaminophen overdose. Collectively, we argue that 16 genes identified in our study are robust mechanism-relevant biomarkers of liver necrosis that may be used to profile toxicity across individuals and in multiple independent microarray studies.

Importantly, the genes identified in this study are interesting not only as potential biomarkers, but also as mediators of acetaminophen-induced cell death and regeneration in liver. For example, the role of OSMR in acetaminophen-induced liver injury deserves attention because genes coding for its two subunits, *Osmr* and *Il6st*, were both identified as genotype-independent biomarkers of the liver toxicity outcome. It is known that IL6ST expression is essential for the control of the hepatic acute-phase

response during liver regeneration [120, 121]. However, while IL6 represents one of the best studied cytokines, there is relatively little known about the biological activities of oncostatin M (OSM), a cytokine secreted by activated T lymphocytes, macrophages, and neutrophils. Oncostatin M may have a pro-fibrotic role in liver injury owing to its ability to induce tissue inhibitor of metalloproteinases (TIMP) 1[122] and TIMP3[123]. While OSM has been shown to be increased following acetaminophen-induced liver injury [124], *Osmr* transcript levels have not been shown previously to correlate with liver necrosis endpoints. Additionally, knockout mice deficient for *Osmr* display defects in liver regeneration following carbon tetrachloride exposure[125]; more importantly, administration of exogenous OSM ameliorated liver injury in wild type mice[125].

In addition, expression of *Mlxipl*, also known as carbohydrate response element binding protein (*Chrebp*), a transcription factor that plays a central role in the dietary regulation of hepatic gene expression by glucose, was decreased as the degree of liver necrosis increased in animals treated with acetaminophen. Several recent studies demonstrated that acetaminophen can affect blood glucose levels[126] and improve glucose tolerance in mice fed a high fat diet[127]. The former study showed that daily administration of acetaminophen prevented approximately 70% of weight gain compared to mice fed the high fat diet alone, even at a daily dose that was lower than half of the maximum recommended weight-adjusted human dose[126]. In addition, decreases in liver glucose and increases in lipid content were observed in the mouse

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liver after acetaminophen overdose using NMR-based metabolomics[128] and may explain the dramatic decrease in *Mlxipl* transcript levels observed in our work. While further studies need to be conducted to link effects on glucose modulation at sub-acute doses of acetaminophen with the acute toxic doses used in our study, changes in *Mlxipl* expression may yield insight into the mechanism of these phenomena.

An important limitation of the animal studies of toxicity mechanisms is the ability to translate the data to clinical findings. A recent rat-to-human study of acetaminophen toxicity showed successfully that gene expression data from peripheral blood cells can provide valuable information about exposure levels, well before liver damage may be detected by classical parameters[16]. The major biological signal in the classifier genes identified in that study was activation of an inflammatory response. None of the 26 genes identified in our multi-strain study could be matched to human blood transcriptome data from subjects hat overdosed with acetaminophen as reported by Bushel *et al.*[16]. However, it should be noted that the small sample size of the human study (5 cases, 2 controls) as well as the wide variability in the timing of the collection of human blood samples, at two or five days after ingestion of acetaminophen, could have been the major factors for lack of mouse-to-human overlap.

F. CONCLUSIONS

The use of toxicogenomics as a tool in toxicology calls for the careful evaluation of study designs. Because one of the major applications of toxicogenomics is to discover biomarkers of toxicity that are relevant to humans, great care must be taken in choosing the appropriate model systems. Traditional risk assessment practices using animal models allow for the control of many experimental factors except for genetics. Although rodent models have been widely used for toxicity testing, their utility is often limited by: (i) inaccurate generalizations from a single genome; (ii) inability to distinguish small and biologically important changes from background variation; (iii) ineffective exploitation of reproducible genetic variation to dissect differential response to chemical exposure; and (iv) inefficient use of defined genetic backgrounds to model particular phenotypic profiles observed in human populations.

To address these important limitations, panels of genetically-defined organisms, such as inbred mouse lines, that provide a fixed genotype within a particular strain but encompass great genetic diversity across strains, are being used more frequently in biomedical research[129]. Inbred mouse strains are reasonably well-suited for identifying whole-genome response signatures indicative of chemical exposure because much is known regarding genetic lineage and derivation for hundreds of strains, and the number and distribution of genetic polymorphisms among mouse strains is equal to or exceeds that in the human population[74, 130]. This approach has the added advantage of "repeat

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testing" in genetically identical individuals within a given strain, yielding important information regarding reproducibility of the response.

Genetic variation among individuals is reflected in variations in gene expression levels[85, 110], which introduces additional challenges into toxicology research. Our recent study to dissect genetic networks that control liver gene expression identified several loci that control the expression of large numbers of genes in the liver[111]. Similarly, the largest group of genes identified in this study as significantly different between individuals, despite the fact that over 2/3 of all animals exhibited variable degrees of liver damage, comprised transcripts that differ in basal levels between inbred mouse strains. Many of the genes with strain-specific expression levels are within receptor activity and G-protein coupled receptor activity molecular pathways. Given the fact that about 50% of all prescription pharmaceuticals currently on the market target this broad class of proteins[131], our data underscores the value of multi-strain experiments that can avert the risk of large genotype, rather than treatment, effects in a particular strain of animals used for pre-clinical safety and efficacy testing.

Table 3.1

Pathway analysis of significantly changed genes

GO ID	GO Class[†]	Function Name	Corrected P-Value
Necrosis			
GO:0004871	MF	signal transducer activity	2.07E-04
GO:0007186	BP	G-protein coupled receptor protein signaling pathway	8.87E-03
Strain			
GO:0004930	MF	G-protein coupled receptor activity	1.60E-05
GO:0004871	MF	signal transducer activity	1.25E-04
GO:0004872	MF	receptor activity	3.42E-04
GO:0004984	MF	olfactory receptor activity	6.27E-04
GO:0001584	MF	rhodopsin-like receptor activity	1.21E-03
GO:0016978	MF	lipoate-protein ligase B activity	2.12E-02
GO:0007165	BP	signal transduction	1.62E-05
GO:0007186	BP	G-protein coupled receptor protein signaling pathway	6.81E-05
GO:0050896	BP	response to stimulus	1.28E-03
GO:0007608	BP	sensory perception of smell	2.59E-03
GO:0008033	BP	tRNA processing	3.98E-03
Strain and Treatment			
GO:0004128	MF	cytochrome-b5 reductase activity	2.75E-02
GO:0004348	MF	glucosylceramidase activity	2.75E-02
GO:0004024	MF	alcohol dehydrogenase activity, zinc-dependent	2.75E-02
Strain and Necrosis			
GO:0008307	MF	structural constituent of muscle	2.05E-02
GO:0005515	MF	protein binding	2.61E-02
GO:0005524	MF	ATP binding	3.61E-02
GO:0005200	MF	structural constituent of cytoskeleton	3.89E-02
Treatment and Necrosis			
GO:0004896	MF	hematopoietin/interferon-class (D200-domain) cytokine receptor activity	7.07E-04
GO:0008538	MF	proteasome activator activity	3.84E-02
GO:0030332	MF	cyclin binding	3.84E-02
GO:0004709	MF	MAP kinase kinase kinase activity	4.79E-02
Strain, Treatment, and Necrosis			
GO:0051010	MF	microtubule plus-end binding	3.67E-03
GO:0008017	MF	microtubule binding	1.56E-02

[†]GO Classes: MF = Molecular Function, BP = Biological Process

Table 3.2

Population-based biomarkers of acetaminophen-induced liver injury

Gene Symbol	Gene Name	Necrosis *P* Value	Treatment *P* Value
DECREASED			
C14ORF122	chromosome 14 open reading frame 122	7.9E-10	3.7E-07
Tlcd1	TLC domain containing 1	1.3E-09	2.4E-07
KIAA1370	KIAA1370	2.9E-09	8.1E-08
Rhbg	Rhesus blood group-associated B glycoprotein	4.3E-09	5.3E-07
Cdc14b	CDC14 cell division cycle 14 homolog B (S. cerevisiae)	1.9E-08	1.1E-07
Lgr5	leucine rich repeat containing G protein coupled receptor 5	2.6E-08	1.6E-07
L2hgdh	L-2-hydroxyglutarate dehydrogenase	3.0E-07	2.1E-07
Mcm10	minichromosome maintenance deficient 10 (S. cerevisiae)	3.3E-07	2.5E-07
Mlxipl	carbohydrate response element binding protein, MLX interacting protein-like	5.9E-07	4.1E-07
INCREASED			
Col4a1	procollagen, type IV, alpha 1	1.1E-13	1.1E-08
Tmem2	transmembrane protein 2	4.8E-12	1.1E-08
Slc39a6	solute carrier family 39 (metal ion transporter), member 6	4.5E-11	2.4E-07
Serpine1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1.3E-09	4.3E-07
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	1.4E-09	7.5E-12
D10Ertd438e	DNA segment, Chr 10, ERATO Doi 438, expressed	4.2E-09	2.0E-09
Psme3	proteaseome (prosome, macropain) 28 subunit, 3	5.5E-08	5.7E-07
Ddx39	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	6.9E-08	2.8E-08
SKIL	SKI-like oncogene	6.9E-08	8.2E-09
Map3k6	mitogen-activated protein kinase kinase kinase 6	7.7E-08	4.6E-07
Pex1	peroxisome biogenesis factor 1	8.4E-08	1.7E-09
Il6st	interleukin 6 signal transducer	2.0E-07	4.3E-08
Osmr	oncostatin M receptor	2.1E-07	2.1E-07
Csf2rb2	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	3.3E-07	1.2E-10
Cd68	CD68 antigen	4.2E-07	3.2E-12
2010109K11Rik	RIKEN cDNA 2010109K11 gene	4.5E-07	4.4E-07
Ipo4	importin 4	6.4E-07	2.9E-07

Figure 3.1

Variability in acetaminophen-induced liver necrosis occurs across mouse strains

Liver necrosis measured across strains after acetaminophen (300 mg/kg, *i.g.*, 24 h) treatment in the left liver lobe shows a gradient of response across mouse strains. Asterisks (*) denote strains that sustained an average of 40% or greater liver necrosis.

Percent Liver Necrosis: 24hr After APAP Treatment

| 0 | 10 | 20 | 30 | 60 | 100 |

Cast/EiJ					
CZECHII/EiJ	PL/J				
NZO/LtJ	WSB/EiJ	SJL/J			
C57L/J	BTBR t+ tf/J	C57BL/6J	BALB/cByJ		
NOD/LtJ	SWR/J	C57BLKS/J	DBA/2J	CBA/J*	
LP/J	A/J	MSM/Ms	JF1/Ms	B6C3F1/J*	
	SM/J	PERA/EiJ	P/J		
	PWD/PhJ	SEA/GnJ	C57BL/10J		
	NON/LtJ	AKR/J	BUB/BnJ*		
	MA/MyJ	FVB/J	KK/HIJ*		
	RIIIS/J	C57BR/CdJ	NZW/LacJ*		
		129S1/SvImJ	C3H/HeJ*		

Rank Order
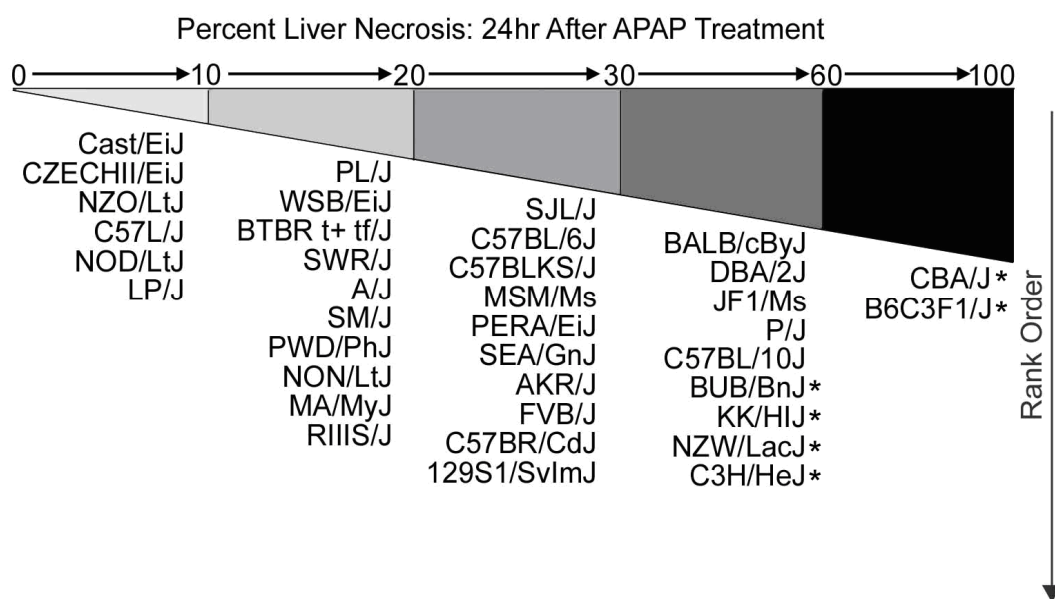
Figure 3.2

Principal components analysis of microarray data

Principal Components Analysis of the global gene expression changes in the left liver lobe following treatment with vehicle (0.5% methyl cellulose) or acetaminophen (300 mg/kg, *i.g.*, 24 h). Acetaminophen-treated samples are depicted as triangles and vehicle-treated samples are depicted as squares. The data separate along the first principal component (PC1) by treatment. There is additional separation of gene expression along PC1 and PC2 by the amount of liver necrosis sustained (white to black scale bar = 0-100% necrosis).
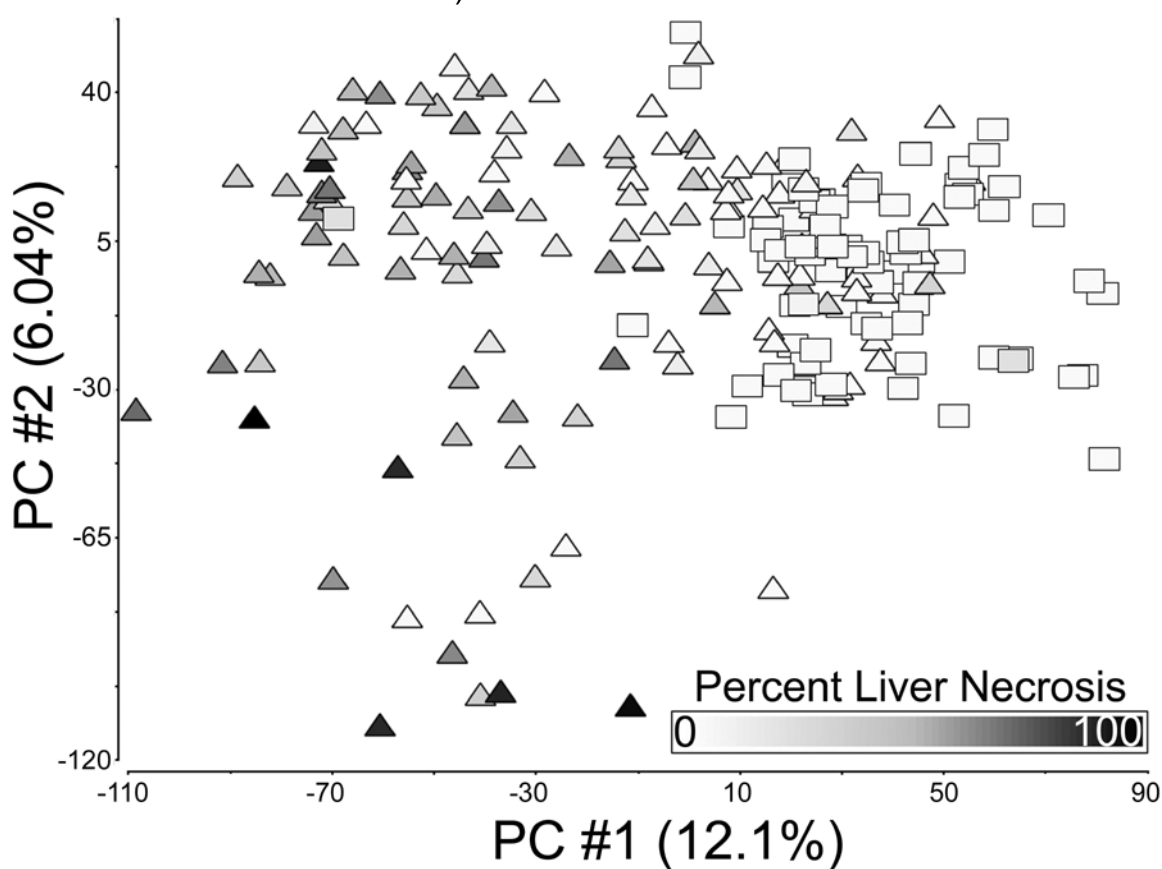
Figure 3.3

Venn diagram of significantly changed genes and heat map of population-based biomarkers of liver injury

(a) The Venn diagram depicts the number of genes significant for each factor in the ANCOVA model, namely treatment, strain (genotype), and the individual's liver necrosis score at 24 h. Population-based biomarkers of response are those 26 genes that are significant for treatment and necrosis score, but not by genotype. (b) The expression patterns of the 26 biomarkers are depicted in a heat map in which samples (rows) were ordered first by necrosis score and then by treatment. Unsupervised hierarchical clustering was performed on heat map genes (columns). (*c*-f) Biomarker gene expression for each sample as plotted against the liver necrosis score is shown for transcript expression that is increased with necrosis: the Oncostatin M receptor subunits *Il6st* (c) and *Osmr* (d) and for transcript expression that is decreased with necrosis: *Mlxipl* (e) and *Cdc14b* (f). Values for vehicle-treated mice are shown in open squares and values for APAP-treated mice are shown in closed circles. The linear regression trend lines for acetaminophen-treated samples are shown.

C *Il6st*

E *Mlxipl*

D *Osmr*

Liver Necrosis

F *Cdc14b*

Liver Necrosis

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Figure 3.4
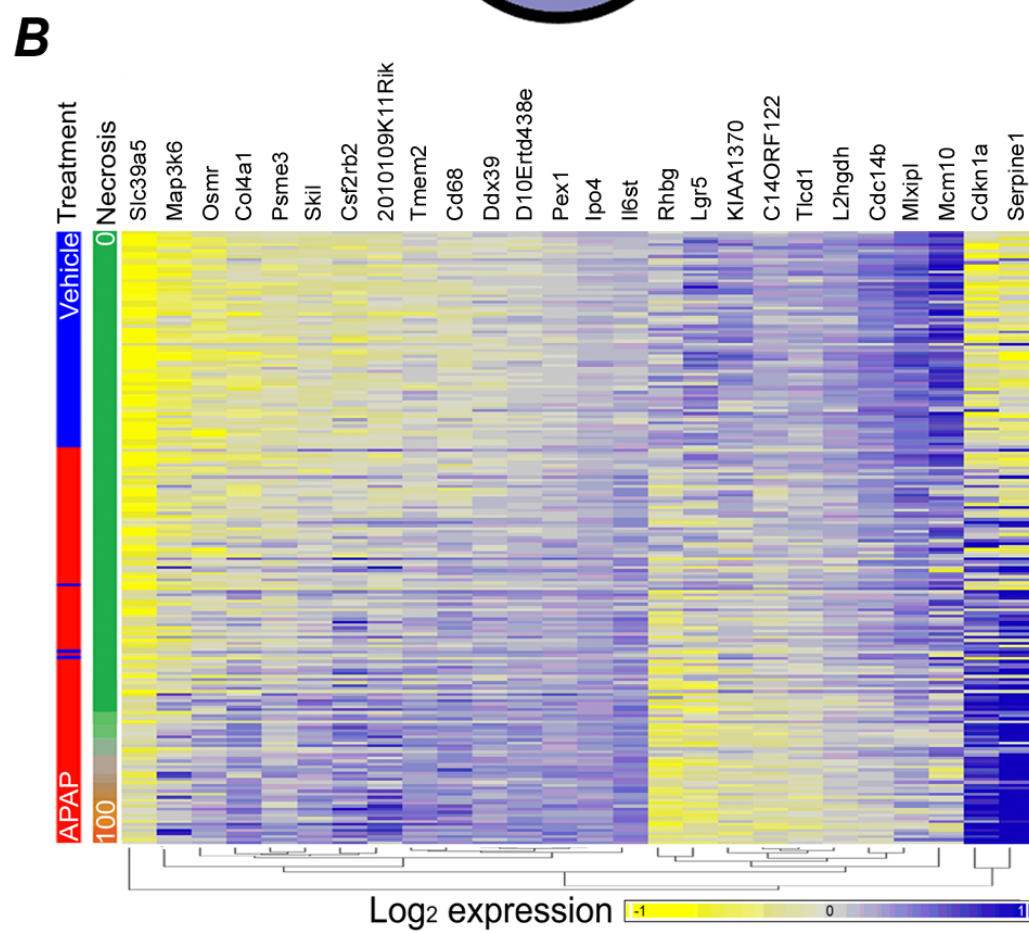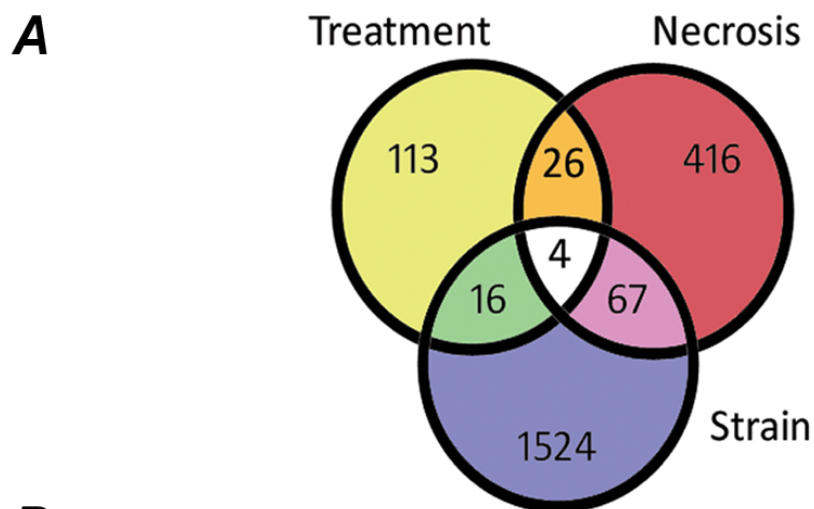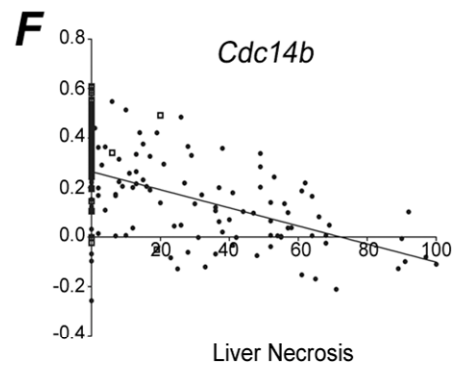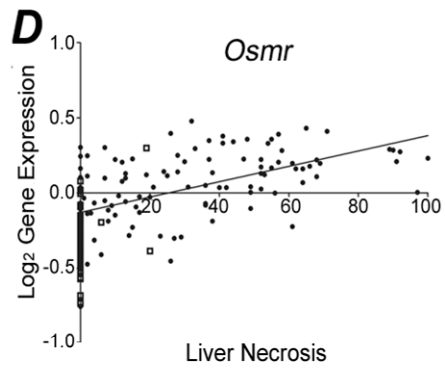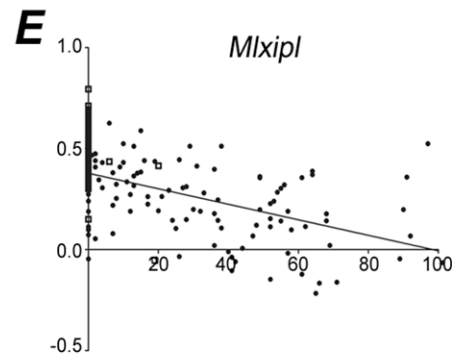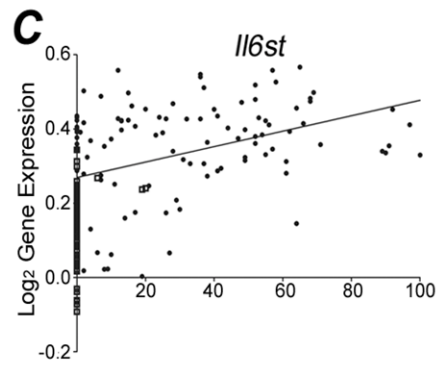
Network analysis of population-based transcript biomarkers

Network analysis of the 26 biomarkers of response using Ingenuity Pathways Analysis revealed that 16 of the biomarkers are closely associated with molecular pathways involved in cell death and proliferation. The network is shown here with transcripts localized to their endogenous sub-cellular compartments (nucleus, cytoplasm, cell membrane, and extracellular space). Genes that are increased or decreased as necrosis increased are colored blue and yellow, respectively.

Chapter 4

OVERALL LIVER TOXICITY OUTCOME DUE TO ACETAMINOPHEN OVERDOSE
IS NOT DUE TO STRAIN-SPECIFIC DIFFERENCES IN ACETAMINOPHEN
METABOLISM

A. ABSTRACT

Research within our laboratory shows that differences in the genetic makeup of particular mouse strains used in acute acetaminophen toxicity studies can have a profound effect on the overall toxicity outcome. Acetaminophen-induced liver injury has a complex etiology that requires: (i) an accumulation of a reactive metabolite, (ii) the activation of *Jun*-mediated intracellular signaling, changes in mitochondrial permeability, and (iii) the subsequent release of chemical mediators that regulate inflammatory mediators which affect the balance of injury and repair within the hepatic parenchyma. Previous research aims successfully identified genetic and transcriptional biomarkers of sensitivity and response to APAP-induced liver injury. However, these approaches did not sufficiently characterize the potential of genetically pre-determined differences in the metabolism of APAP across strains to contribute to the overall toxicity outcome. Increases in the ability to convert APAP to its reactive metabolite, NAPQI, via cytochrome P450 metabolism, or decreases in the ability to detoxify metabolites downstream of NAPQI formation could result in increased susceptibility to liver necrosis that may, in part, explain the sensitivity of some strains (Figure 4.1). Here we characterize APAP metabolism within five inbred strains utilized in previous studies that demonstrated a genetically-determined range of liver toxicity outcomes. By measuring the major metabolites of APAP in serum and by assessing key pharmacokinetic parameters, we determined that the contribution of APAP metabolism to the liver toxicity

outcome, while important to the etiology, is not the determining factor in the overall APAP-induced liver injury outcome that is genetically-determined between tested strains.

B. INTRODUCTION

It is well-accepted that genetic sequence variations can affect an individual's response to drug treatment[21, 22]. As early as the 1970's, researchers noted individual differences in drug efficacy; for example, the daily dose of warfarin needed to achieve a similar degree of anticoagulation in 200 patients was shown to vary widely[132]. Variability in drug response also applies to adverse side effects, especially when the adverse side effects relate to the inherent pharmacologic properties of the drug, such as in the use of chemotherapeutics to treat cancer. However, it is difficult during drug development to predict rare, "idiosyncratic" adverse events that are only detected when thousands or millions of people are administered a given drug.

Research into the identification of genetic variants that predict therapeutic efficacy or that confer an increased risk of adverse effects is increasing, as the FDA recently began allowing drugs to remain on the market based on the availability of genetic tests. Due to recent successes in genetic testing for drugs such as warfarin[28, 29] and 6-mercaptopurine[26, 27], there is a significant demand for the development of models that can efficiently identify genetic sequences that affect pharmacogenetic traits. Recent

studies within our laboratory and others have shown that the genetic background of a particular mouse strain can determine an inherent susceptibility to acetaminophen-induced liver necrosis[133, 134]. We therefore chose APAP-induced liver injury as a model exposure for use in an inbred mouse strain panel in order to validate an approach that identifies sequence variants that influence the liver toxicity outcome following an acute dose. An increase in susceptibility was found to be associated with genetic variability within the *Cd44* gene in both mouse and human test cohorts.

Acetaminophen is highly metabolized within the body; with only 2-5% of the dose excreted unchanged in the urine[135]. The major metabolites of acetaminophen are the sulfate and glucuronide conjugates, but a minor fraction is biotransformed primarily within the liver by cytochrome P450 enzymes to an electrophilic quinone radical, NAPQI. At therapeutic doses, NAPQI is inactivated rapidly by glutathione conjugation and excreted as cysteine and mercapturic acid conjugates (Figure 4.1). However, large doses of acetaminophen result in a depletion of hepatocellular glutathione that leads to covalent binding of excess NAPQI with cellular macromolecules, eventually causing cell death by necrosis[77]. In early studies, we demonstrated that levels of hepatic GSH, which contributes to the detoxification capacity of the liver for NAPQI, were different between strains at 4 h after dosing, but not correlative with overall necrosis outcome. However, it was not known whether the overall pharmacokinetics of APAP, which is a key factor to the progression of injury, differed between strains.

Because the reactive metabolite is formed in parallel with the sulfate and glucuronide conjugates[136], the kinetics of formation of the conjugates affect the extent and timing of reactive metabolite formation (thereby influencing the exposure to APAP), and the liver injury outcome of acetaminophen toxicity may be affected. It was therefore possible that resistant strains might have an enhanced capacity to clear the parent drug through sulfation and glucuronidation pathways. In this study, we did not consider the absorption of orally dosed APAP to be a major factor in strain differences, because it has been previously demonstrated that absorption from the gastrointestinal tract is mediated by passive transport[137] with a negligible amount of metabolism within the gut mucosa (shown in rats)[138]. Our study instead focused on assessing the formation and elimination kinetics of acetaminophen metabolites in order to determine whether strain differences play a role in genetic sensitivity to acetaminophen-induced liver toxicity.

C. MATERIALS AND METHODS

Animals and treatments

Adult (aged 6-8 weeks) male mice of strains C3H/HeJ, C57BL/6J, DBA/2J, LP/J, and NZW/LacJ were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were fed with a commercial NTP-2000 wafer feed (Ziegler Brothers, Inc., Gardners, PA) and water *ad libitum* and maintained on a standard 12 h light-dark cycle. Mice from which plasma metabolite levels were measured were singly housed in polycarbonate cages

with Sani-Chips irradiated hardwood bedding (P.J. Murphy Forest Products Corp., Montville, NJ). For the 300 mg/kg dose and vehicle controls, mice were fasted for 18 h before the start of dosing. At 9 am, animals were dosed (*i.g.*) with 50 mg/kg or 300 mg/kg acetaminophen (APAP; Sigma-Aldrich, St. Louis, MO), or with the 0.5% methylcellulose (Sigma-Aldrich) vehicle in a 10 ml/kg dosing volume. Food was returned at 3 h post-dosing.

Serum and tissue collection

Serum collection. Blood (45 ul) was collected sequentially from the tail vein at 0, 0.5, 1, 2, and 3 h post-dosing. At 6 h, mice (N=5 per strain) were administered Nembutal anesthesia (100 mg/kg *i.p.*, Abott Laboratories, Chicago, IL). Blood was collected by exsanguination at 6 h for metabolite measurements and ALT quantification. Blood samples were then centrifuged and serum was stored at -20ºC until high-performance liquid chromatography (HPLC) analysis.

Tissue collection. Livers were quickly excised following ex-sanguination and sections of the left and median lobes were placed in 10% phosphate buffered formalin (Sigma-Aldrich) for immunohistochemical analyses.

Acetaminophen metabolite quantification

Metabolite measurements in serum. The procedure used for the quantification of APAP and its major metabolites, the glucuronide and sulfate conjugates (AG and AS), is similar to that previously described[139]. Standards for each analyte were obtained from

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Sigma-Aldrich. Briefly, a reversed-phased HPLC assay was used in which the mobile phase was 5% acetonitrile and 95% 5 mM sodium sulfate/20 mM potassium phosphate buffer (pH=3.2) with a flow rate of 1.2 ml/min. Retention times for APAP, APAP-G, APAP-S, and the internal standard (3-acetaminophenol; Sigma-Aldrich) detected at 254 nm were 3.6, 5.2, 7 and 11 min respectively.

The $AUC_{0-\infty}$ was calculated by using noncompartmental analysis in WinNonLin (Pharsight, Mountain View, CA). A one-way ANOVA with a Tukey post-hoc test was used to assess significantly different AUC across mouse strains ($P<0.05$).

Liver necrosis assessment

To determine the overall liver injury outcome from acute APAP dosing experiments, a point scoring technique was utilized as described in Chapter 2 (Methods). Liver injury, assessed within the left liver lobe, was expressed as the percent of necrotic area in a 100X field relative to non-necrotic areas in the same field.

Quantification of serum alanine aminotransferase (ALT)

The quantification of ALT in mouse serum was performed as previously described[82]. Briefly, serum ALT was quantified using a kinetic method in which the rate of oxidation of NADH by lactate dehydrogenase (LDH) is measured at 340 nm following the addition of ALT (GPT) Reagent (Thermo Fisher Scientific, Waltham, MA) per the manufacturer's recommended procedure.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (5µm) of the left liver lobe were mounted onto glass slides. Immunostaining was performed as previously described[10], using the DAKO EnVision system HRP (Dako Cytomation, Carpinteria, CA) with primary antibody (1:200 nitrotyrosine [Molecular Probes, Eugene, OR]) and counterstaining was performed with hematoxylin. To quantitatively measure staining, all slide sections were processed in parallel on the same day. Quantitative measurements of antibody staining were performed using Image Pro Plus software (version 5.1; Silver Spring, MD). Briefly, the percent area stained to the total area within pericentral regions was determined for each animal by averaging the data from five areas per slide (at 400X magnification).

Determination of glutathione measurements in liver tissue

Reduced and total glutathione amounts in liver were determined using the ApoGSH Glutathione Colorimetric kit (BioVision, Mountain View, CA). Briefly, 50 mg of frozen tissue from the left liver lobe was homogenized in Glutathione Buffer™ and centrifuged for 10 min at 8000 x *g* following the addition of 100 µl 5% sulfosalicyclic acid. The supernatant was then assayed for reduced and total glutathione according to the manufacturer's protocol. Glutathione levels were quantified using the pseudo-end point method based upon a generated standard curve.

Enzyme-linked Immunosorbent Assay (ELISA)

Isolation of microsomes. Microsomes were isolated from the left liver lobe (300 mg/kg APAP; 24 h) by homogenizing 80 mg tissue in 240 µl ice-cold microsome buffer (50 mM Tris-HCl pH 7.4, containing 150 mM KCl, 1 mM EDTA, protease inhibitor mix, and 20% (v/v) glycerol). Homogenates were centrifuged at 9000 x g for 20 min at 4ºC. The supernatant was then centrifuged at 105,000 x g for 60 min at 4ºC. Microsomal pellets were resuspended in microsome buffer. Protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific Inc., Rockford, IL) and the manufacturer's protocol.

Protein quantification. Quantitative determinations of protein levels of cytochrome p450(CYP) 2E1, CYP1A2, catalase, and glutathione S-transferase (GST) in liver microsomes were performed by Integrated Laboratory Systems, Inc. (RTP, NC). Protein levels were determined by using the Protein Detector ELISA kit protocol (KPL, Inc. Gaithersburg, MD) as detailed by the manufacturer. ELISAs were performed by Integrated Laboratory Systems, Inc. (Research Triangle Park, NC).

D. RESULTS

Variability in acetaminophen-induced liver injury exists among mouse strains

We characterized the potential for acetaminophen to cause varying degrees of liver injury in the five inbred strains by measuring ALT in serum as an indirect measure

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of hepatocellular necrosis. ALT levels were assessed at 6 h following administration of a 50 mg/kg dose and at 6 and 24 h following a 300 mg/kg dose of APAP under standard housing conditions. At the sub-toxic dose, we observed minor differences in ALT levels across strains in which strain LP/J exhibited a lower ALT level than all other strains at 6 h (Table 4.1). However, these data for the sub-toxic dose are not considered indicative of liver injury because the ALT levels in APAP-treated mice are not significantly increased with respect to vehicle controls. At 6 h following the acute dose, ALT levels in serum were significantly elevated with respect to controls for all tested strains. However, there was no difference in the ALT elevations among the five inbred strains that would explain differences in liver necrosis outcome observed histologically at 24 h under the same experimental conditions (P=0.0714, Table 4.1).

Protein levels of APAP metabolic enzymes do not correlate with susceptibility to liver toxicity

In order to assess the contributions of APAP metabolism to the liver toxicity outcome, levels of a few key metabolic enzymes were assessed in the livers of mice from each strain. Protein measurements were performed by ELISA from microsomes derived from the left liver lobe of mice treated with 300 mg/kg APAP and necropsied at 24 h. Linear regression analysis was performed to determine whether there was a tendency for the amounts of catalase, Cyp2E1, Cyp1A2, and Glutathione *S*-transferase (GST) Pi enzymes to correlate with the percent liver necrosis at 24 h (Table 4.2). There

was no significant relationship detected between the amounts of these enzymes with necrosis at either the basal level (vehicle-treated) or the induced level (APAP-treated) in these mice.

APAP metabolite profiles in plasma do not correlate with liver injury outcomes

We next investigated potential strain-based differences in the kinetics of APAP metabolism by measuring serum concentrations of APAP and its two major metabolites. APAP, APAP-glucuronide (AG), and APAP-sulfate (AS) concentrations were measured for the subtoxic and toxic doses from time 0 to 6 h. Serum was extracted from individual mice across the entire time course as described in Methods so that the area under the curve (AUC) of each profile was derived from a single individual.

There was no difference in the exposure to APAP or the AUC of the glucuronide and sulfate conjugates at the 50 mg/kg dose level between strains. However, we found a significant main effect of strain in the AUC of APAP, AG, and AS at the 300 mg/kg dose level (P=0.006, P=0.0025, and P=0.0003 respectively). These data were presented in Figure 2.3. Interestingly, the resistant strain, LP/J, exhibited the highest AUC of the glucuronide and sulfate conjugates and tended to have higher concentrations of these metabolites in the serum than other strains at 3-6 h post-dosing (Figure 4.2). However, data from strain LP/J also demonstrated a much greater average exposure to the parent drug.

We next examined the relationship between metabolite AUC and the liver necrosis outcome (24 h) after the 300 mg/kg dose (Figure 4.3). As expected, a greater AUC of the glucuronide conjugate was associated with lower liver necrosis (P=0.0019, R^2=0.9731). However, there was also a significant association between APAP exposure and liver necrosis, in which strains with a greater exposure to the parent compound had a lesser degree of liver injury (P=0.0019, R^2=0.8291). There was no significant association observed between the AUC of the sulfate conjugate and liver necrosis (P=0.077, R^2=0.7007).

Hepatic glutathione levels at 6 hours are not associated with liver injury outcome

In order to determine whether the detoxification capacity for the reactive metabolite differed amongst strains and was associated with injury outcome, we measured levels of total glutathione and GSH in livers of mice at 6 h. There was found to be no difference in total glutathione by either strain or treatment (P=0.2269 and P=0.4234; Figure 4.4A). While there was a difference in strain (P=0.0014) and treatment (P=0.0001) in the levels of hepatic GSH (Figure 4.4B), the difference was associated with a significant interaction between strain and treatment (P=0.0131). Interestingly, the levels of total glutathione, but not of GSH, in the vehicle-treated animals were found to be correlated with the 24 h percent liver necrosis across strains (P=0.015, R^2=0.8948). However, this observation is likely not a meaningful association because reduced

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glutathione, rather than the oxidized form, is the chemical entity responsible for detoxification of the reactive metabolite. Taken together, the data do not indicate that the level of total or reduced glutathione that differs between strains (when measured at 6 h) has a significant impact on the liver necrosis outcome following a toxic APAP dose.

Nitrotyrosine adducts are significantly lower in APAP-resistant strain LP/J

To assess the ability of strains to produce and detoxify NAPQI, we first attempted to measure APAP-protein adducts by immunostaining using a commercially available antibody. However, staining tests with this antibody were unsuccessful and it was later revealed by the manufacturer that the antibody had insufficient quality control.

We then indirectly measured the level of oxidative stress experienced in the liver through the detection of peroxynitrite via nitrotyrosine antibody staining. The resistant strain LP/J exhibited the lowest level of nitrotyrosine staining which was significantly different from all other strains, indicating a lesser degree of oxidative stress experienced by mice of this strain (P=0.0012; Figure 4.5). However, the large degree of necrosis and erythrocyte infiltration into the hepatic parenchyma present for all tested mouse strains may play a role as a confounding factor that contributes to nonspecific binding of the anti-nitrotyrosine antibody that can complicate accurate quantification.

E. DISCUSSION

We previously demonstrated that the genetic background of a particular mouse can affect the overall toxicity outcome following an acute dose of acetaminophen. Subsequent to the toxicity studies, we determined genetic biomarkers that affect an individual's susceptibility to liver injury (Chapter 2), as well as gene transcript biomarkers that correlate with the liver necrosis outcome (Chapter 3). An interesting result of these early studies was that none of the biomarker genes identified is known to be involved in the metabolism of acetaminophen in the liver.

In parallel with our previous studies, the results of this study did not demonstrate a clear association with acetaminophen plasma pharmacokinetic parameters and the liver injury outcome in genetically susceptible and resistant mouse strains. In particular, we did not determine a difference between strains in the ability to form the reactive metabolite, NAPQI. There was no evidence for variability in the amounts of CYP2E1 and CYP1A2, the enzymes responsible for the bioactivation of acetaminophen to the reactive, injury-causing metabolite, NAPQI. While there was some evidence that the resistant strain, LP/J, experienced a relatively lower amount of ROS generation, the data are difficult to interpret due to the extensive liver necrosis that may contribute to non-specific binding of the probe antibody for nitrotyrosine.

It is interesting that the AUC of the sulfate and glucuronite conjugates of strain LP/J was also significantly greater than that of the other four strains. In a study that

investigated pharmacokinetic differences between rat strains that are resistant (Sprague-Dawley; SD) and susceptible (Long Evans Hooded; LEH) to acetaminophen-induced liver injury, it was noted that the resistant strain had a greater capacity for glucuronidation and sulfation of the parent compound[140]. Of these effects, the increased capacity for glucuronidation was found to be the major component that contributed to increased injury resistance[140]. However, in these studies using a rat model, unlike in our mouse studies, the increased resistance was accompanied by enhanced levels of hepatic glutathione (GSH)[141]. Additionally, the mechanisms by which sulfation is limited has been shown to differ in rats and mice. In rats, sulfation is limited by the availability of 3'-phosphoadenosine 5'-phosphosulfate (PAPS; *i.e.* the cosubstrate for sulfation); In CF-1 mice, PAPS is not depleted at a dose of 600 mg/kg acetaminophen[142] and sulfation is instead limited by hepatic sulfotransferase activity[143]. Therefore, it is reasonable to speculate that the increased sulfation and glucuronidation capacity of the LP/J mouse may play a role in its resistance to acetaminophen-induced toxicity and may be related to a strain-dependent enhancement of sulfotransferase activity. However, further characterization of this mechanism is needed.

While there were no observable differences in GSH levels across mouse strains, it is not possible from the data collected to determine whether there were strain-dependent differences in the maximum GSH depletion after acetaminophen administration. A confounding factor is the measurement of GSH at 6 h following

treatment rather than at 2 h when the maximum depletion has been shown to occur[144, 145]. In order to fully characterize the ability of each strain to detoxify NAPQI via GSH conjugation, it would be beneficial to collect livers over time and conduct measurements from time 0 to 6 h. To address whether strain-dependent differences occur in this pathway, glutathione conjugates were measured in urine collected for 24 h post-dosing by NMR, however the data was not yet available to be included in this manuscript.

The data from our study do not demonstrate a clear correlation between acetaminophen pharmacokinetics and downstream toxicity; this is not surprising in light of previous multi-strain studies that examined the pharmacological effects of acetaminophen. In a previous study that examined the role of genetic differences to affect acetaminophen's antinociceptive effects, 12 inbred mouse strains were administered a writhing test for pain responses following administration of a 150 mg/kg (*s.c.*) dose[146]. It was noted that there was a great deal of variability across strains and, in particular, C57BL6/J and DBA/2J mice were found to be susceptible and resistant to the antinociceptive effects, respectively. Assessment of plasma acetaminophen concentrations over a two hour time course demonstrated that there was no difference in the AUC of APAP between the two strains. In addition, there was no difference in the clearance, the absorption rate constant, or the volume of distribution observed for these two strains. Although the precise mechanism of acetaminophen's antinociceptive ability is still debated[147], it appeared from this study that, since the strain-dependent hot-plate

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antinociception was not accompanied by evidence of strain-dependent pharmacokinetic parameters, the relevant genes governing the response are more likely involved in a pharmacodynamic role.

There are additional pharmacodynamic parameters that may underlie a genetic basis for strain susceptibility that were not examined in this study, which include hepatic drug transport processes. It has been shown in mice that acetaminophen can alter hepatocellular transport processes by causing a temporal down-regulation in mRNA expression of hepatic uptake carriers (*Oatp* and *Ntcp*) and an up-regulation of *Mrp* efflux and stress genes (*Ho-1* and *Nqo1*)[148]. The authors of this study hypothesized that the liver altered gene expression of these transporters following liver injury in order to limit the accumulation of harmful chemicals within the hepatocyte[148]. The uptake of acetaminophen into hepatocytes has been shown to be accomplished by passive diffusion, rather than active transport processes[149]. Therefore, it is likely that the changes in transporter expression reflected an adaptive change by which the hepatocyte could more easily export acetaminophen metabolites upon a second challenge or prolonged exposure. In our study, the increase in serum glucuronide and sulfate conjugates observed for resistant strain LP/J may reflect strain-dependent differences in transport protein abundance or function rather than a greater capacity for these phase II reactions. For example, rat models have demonstrated that phase II conjugates formed in hepatocytes can be secreted into sinusoidal blood, rather than into bile, if there is an

impairment of the MRP2 transporter[150]. However, since the ratios of each metabolite to parent AUC do not differ between strains (P=0.17, AS; P=0.21, AG), it is unlikely that there are strain-dependent differences in the capacity for sulfation and glucuronidation pathways for acetaminophen.

F. CONCLUSIONS

In summary, the data do not conclusively support a role for strain-dependent pharmacokinetics to determine the overall liver injury outcome following an acetaminophen overdose. The data are not surprising, given the complexity of cellular responses that have been demonstrated to modulate acetaminophen-induced liver injury. It is more likely that processes downstream of metabolism, such as the *Jun*-mediated cell death cascade[151] and cross-talk between hepatocytes and innate immune cells[56, 152] have a greater effect on strain-dependent toxicity outcomes. This study underscores the need for toxicologists to adopt genetically heterogeneous mouse models in which global genomics approaches for novel biomarker discovery can be used. The model may prove especially useful for pharmaceutical risk assessment in which traditional toxicological parameters do not predict the individual toxicity outcome.

Table 4.1

Liver injury measured in susceptible and resistant strains

Summary of serum ALT and percent liver necrosis measured across inbred mouse strains at 6 and 24 h following a subtoxic (50 mg/kg) or toxic (300 mg/kg) dose of APAP (mean ± S.E.) under standard housing conditions. *P* values were derived from one-way ANOVA analysis across strains.

	LP/J	C57BL/6J	DBA/2J	NZW/LacJ	C3H/HeJ	*P* value
0 mg/kg	56 ± 34	36 ± 8	30 ± 0	64 ± 36	31 ± 1	0.6506
6 h ALT						
50 mg/kg	28 ± 2	41 ± 2	45 ± 6	40 ± 5	56 ± 8	0.0199
300 mg/kg	3101 ± 547	3413 ± 754	3207 ± 364	2691 ± 549	2823 ± 708	0.9207
24 h ALT						
0 mg/kg	56 ± 34	36 ± 8	30 ± 0	64 ± 36	31 ± 1	0.6506
300 mg/kg	2992 ± 1643	4303 ± 567	5057 ± 105	5830 ± 243	5087 ± 539	0.0714
24 h Necrosis	11 ± 9	22 ± 13	34 ± 8	46 ± 16	55 ± 11	0.1387

Table 4.2

Liver acetaminophen metabolic enzyme levels determined by ELISA

Levels of metabolic enzymes involved in APAP metabolism and detoxification were measured in livers of mice treated with 300 mg/kg APAP or vehicle and necropsied at 24 h post-dosing (mean). R^2 and P values were determined for the linear regression between enzyme amount and the percent liver necrosis score for each strain.

	LP/J	C57BL/6J	DBA/2J	NZW/LacJ	C3H/HeJ	R^2	P value
APAP							
Catalase	394	658	574	613	702	0.57	0.14
CYP1A2	146	190	177	161	161	0.0004	0.97
CYP2E1	89.5	90	93.5	80.3	74.6	0.63	0.11
GST Pi	762	1230	680	405	1020	0.034	0.77
Vehicle							
Catalase	461	900	800	411	743	0.0013	0.95
CYP1A2	90.3	312	230	182	167	0.0023	0.94
CYP2E1	95.8	212	132	147	80.6	0.077	0.65
GST Pi	468	1680	1570	707	704	0.02	0.82

Figure 4.1

Acetaminophen metabolism scheme

Percentages represent the amount of the total acetaminophen dose metabolized by each pathway at therapeutic doses in humans. The figure is adapted from Nelson and Bruschi[153].

Acetaminophen glucuronide
45-60%

Acetaminophen sulfate
25-30%

Acetaminophen
5%

N-acetyl-*p*-benzoquinone imine
(NAPQI)
5%

3'-(*S*-glutathionyl)-acetaminophen

NAPQI bound to proteins and
cellular macromolecules

3'-(*S*-cysteinyl)-acetaminophen

3'-(*S*-mercapto)-acetaminophen

CELL DEATH
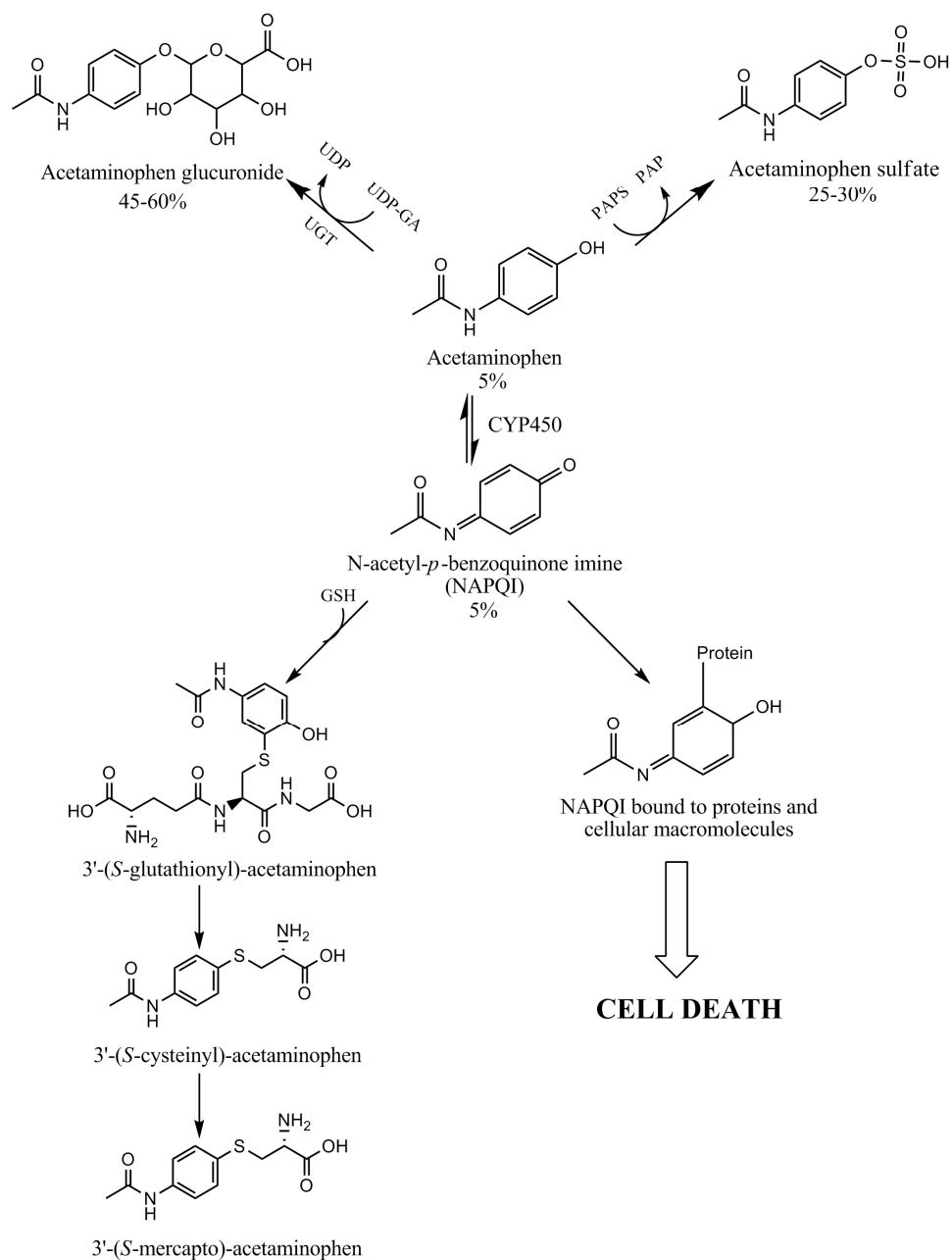
Figure 4.2

Plasma concentrations of APAP, APAP-glucuronide, and APAP-sulfate

Concentrations of APAP and glucuronide and sulfate conjugates in serum are shown for a 50 and 300 mg/kg dose across each strain from 0 to 6 h post-dosing. Points on the curve represent mean ± S.E. (N=4-5 mice per strain).
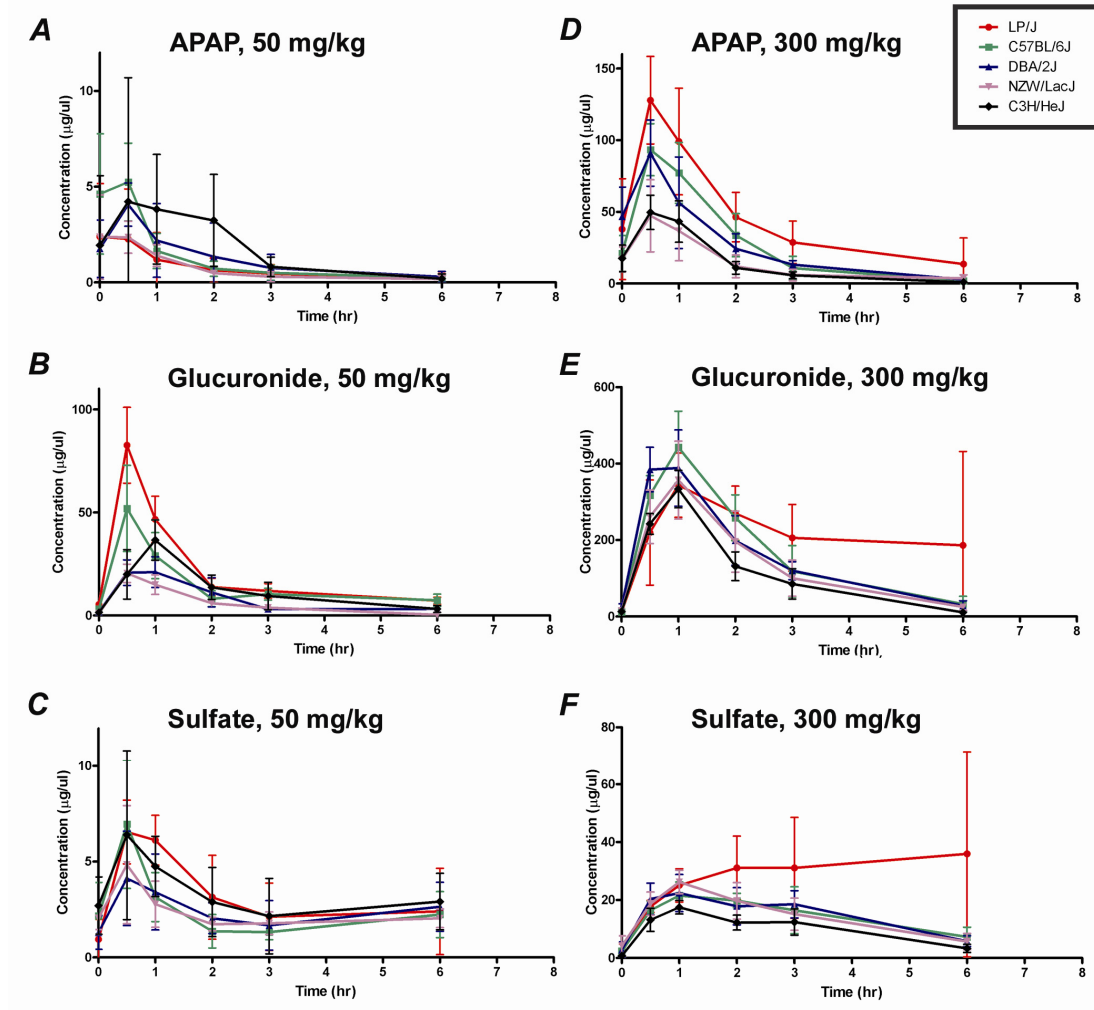
Figure 4.3

Linear regression analysis of plasma metabolite concentration with mouse strain liver necrosis

Linear regression analysis for the calculated AUC at 300 mg/kg of APAP and the glucuronide (AG) and sulfate (AS) conjugates with percent liver necrosis. Points represent mean values per strain and dotted lines represent the 95% confidence interval of the regression line.
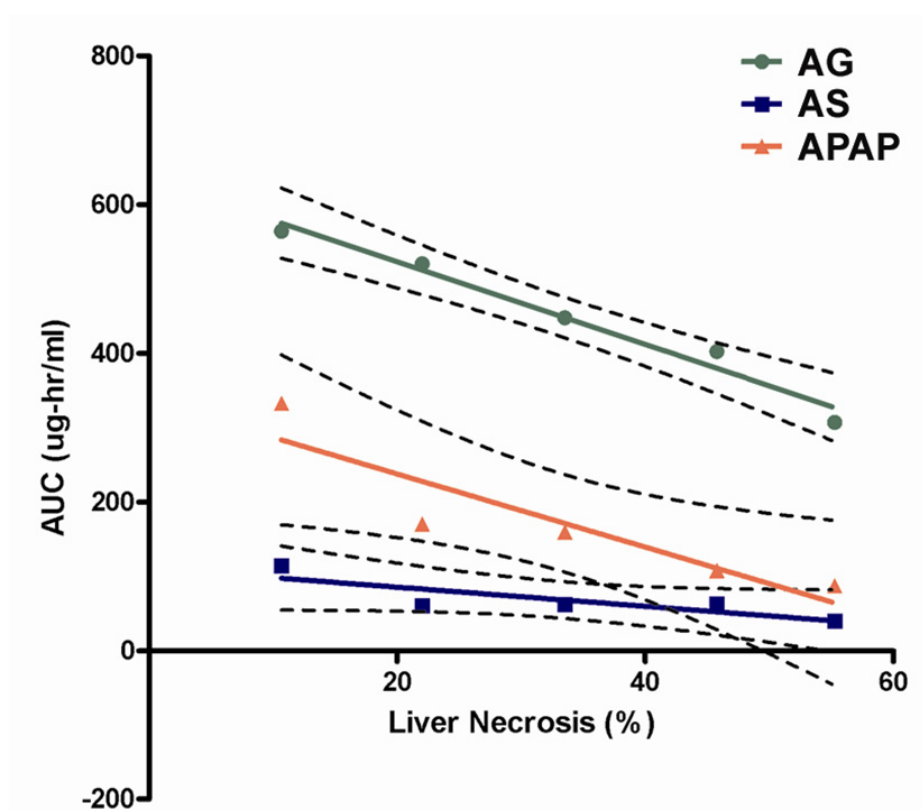
Figure 4.4

Liver total and reduced glutathione levels in susceptible and resistant strains

Total and reduced (GSH) glutathione levels measured in livers extracted from mice necropsied 6 h after a 300 mg/kg dose of APAP or 0.5% methylcellulose vehicle (mean ± S.E.).
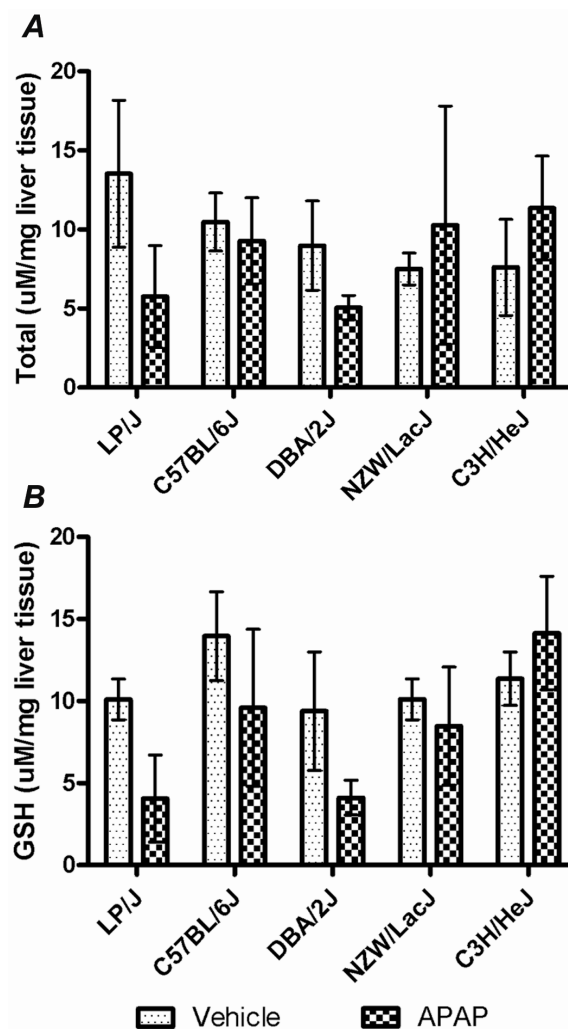
Figure 4.5

Liver nitrotyrosine adducts measured in susceptible and resistant strains

Nitrotyrosine adducts measured around the central veins at 6 h post-dosing in the left liver lobes of mice treated with 300 mg/kg APAP (mean ± S.E.).
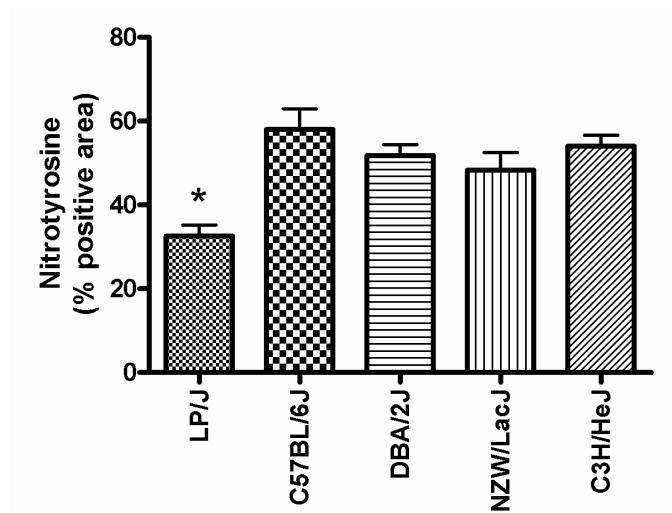
Chapter 5

DISCUSSION

A. CONCLUSIONS AND PERSPECTIVES

Adverse drug reactions are a significant safety concern in drug development and therapeutic implementation because rare adverse effects are often detected late in the drug development process, and occasionally after marketing to the public. Decades of pharmacogenetic research has demonstrated that the genetic make-up of an individual can affect both the efficacy and pharmacokinetics of a drug, as well as confer a propensity for experiencing toxic effects. The standard rodent screening tests used by both industry and government agencies for risk assessment are insufficiently designed to predict rare adverse health events because they employ a single inbred or outbred line that does not represent the genetic diversity present within human populations. Therefore, we developed and tested a "Mouse Model of the Human Population (MMHP)" in order to demonstrate the utility of using a genetically diverse panel of inbred mouse strains to understand and predict liver toxicity in humans. By combining classical toxicological endpoints with rodent genetics, we demonstrated the utility of using the MMHP to model human toxicity responses to acetaminophen (APAP) and to uncover novel biomarkers of liver injury.

1.) Genetic Markers that Predict Toxicity Susceptibility

Recently compiled databases on genetic polymorphisms in many inbred mouse strains afford an unprecedented opportunity to uncover markers that may predict an

inherent genetic sensitivity to toxicant-induced injury. This wealth of information, coupled with the ability to associate phenotypes with genetic haplotypes *in silico*[40, 43] allows for the discovery of genetic loci that affect toxicity phenotypes. As an important first step, we demonstrated that a panel of inbred mouse strains will exhibit a range of liver toxicity after acetaminophen ingestion that is reminiscent of the gradient of toxicity observed in human studies[52]. Using data from an independent cohort of human volunteers, we also demonstrated an inbred mouse strain panel could be used to model the range of liver toxicity previously observed following a subchronic dosing regimen using therapeutic doses of Tylenol[52]. In this study, we identified regions of the mouse genome that contained genetic polymorphisms that correlate with the liver toxicity outcome. One gene, *CD44*, was found to contain a non-synonymous coding SNP associated with an increased tendency in humans to have elevated serum ALT levels during Tylenol exposure. *In silico* modeling suggested that this genetic variant may have functional consequences for the expressed proteins in human. As further evidence that CD44 is able to modulate the liver injury outcome following an acute acetaminophen exposure, we demonstrated that *Cd44* gene knockout mice sustained a greater degree of liver injury than their wild type counterparts (Figure 2.6).

Taken together, the approach serves as an important validation of the MMHP to the prediction of genetic variants affecting toxicity in humans. The ability of the MMHP to detect toxicogenetic loci should lead to an improvement in the safety profile of many

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drugs that have the potential to cause rare, but serious, side effects by facilitating development of improved clinical testing practices. In addition, the use of the MMHP in drug development may lead to a "rescue" of promising drug candidates that have been previously unmarketable due to a toxic side effect in a small proportion of humans enrolled in clinical trials.

We demonstrated that the genetic make-up of the individual can have a marked effect on the toxicity outcome. From a toxicological risk assessment standpoint, the MMHP models the range of human responses and highlights the need to use a panel of strains, rather than a single strain, to extrapolate possible genetic risk in the human population.

2.) Determining Population-Based Biomarkers of Liver Injury

High throughput microarrays provide a sensitive assay to examine molecular changes and responses to a chemical exposure or toxic insult[10, 11, 154]. While previous studies had indicated that acetaminophen exposure in rats and mice can yield information on gene expression changes that occur throughout the timeline of liver injury[11, 133], these early studies failed to capture the diversity of responses that can occur among a population of genetically heterogeneous individuals because they were limited to either a single or only a few inbred rodent strains. By limiting the genetic diversity within the exposure model, the data gathered from these studies may be useful to study

the mechanism of acetaminophen-induced liver injury, but does not provide a robust biomarker that indicates the amount of injury sustained on an individual basis.

To address this experimental limitation, we used the MMHP to derive gene transcript biomarkers of response and determined 26 genes for which expression was altered according to the level of liver injury experienced by the individual. For each biomarker, the expression was independent of the individual's genetic background. This study demonstrated that the model could be a useful tool for correlating a continuous toxicity phenotype with gene expression changes. Perhaps not surprisingly, we identified more than 1500 genes that were changed solely on the basis of mouse strain; these genes may contribute to identification of "false positive" results within single strain studies. This result highlights the importance of using a multi-strain model for toxicity risk assessment because it is clear that the genetic background can have a large impact on basal gene expression, which may confound the data obtained from sensitive microarray studies.

In assessing the mechanism of acetaminophen-induced liver toxicity, the transcript biomarker data support a model in which an apoptotic signaling cascade, centered on Cdk inhibitor p21 (*Cdkn1a*), leads to hepatocellular necrosis. Notably, the biomarker data also implicated a few genes to be involved in the necrotic response that had not been previously linked to acetaminophen-induced liver toxicity. These novel genes included: i) *Osmr,* a receptor for the oncostatin M cytokine, which had previously

been shown to affect liver regeneration following carbon tetrachloride poisoning[125], and ii) *Mlxipl* (also known as *ChREBP*, carbohydrate response element binding protein), an interesting gene given the recent body of research that demonstrates that acetaminophen can affect blood glucose levels[126, 127]. Taken together, the discovery of new acetaminophen-responsive genes supports the idea that using a population-based approach with a continuous phenotype to discover gene expression biomarkers can yield important new information that cannot been obtained from conventional approaches.

The major utility of the approach lies in the ability to quickly assess biomarkers of response within an exposed population. A remaining challenge is to demonstrate that the approach can generate clinically useful biomarkers in a less invasive tissue, such as in peripheral blood. In addition, it would be beneficial to be able to compare the mouse strain dataset to additional human datasets, which were not immediately available, in order to demonstrate a translational validation for the approach. Follow-up analysis in the context of relevant human data is crucial in order to validate the model as a useful technique for accurately assessing human health hazards.

3.) Assessing the Role of Potential Strain-Dependent Differences in Acetaminophen Metabolism to Affect the Liver Injury Outcome

The well-established mechanism of liver toxicity due to acetaminophen overdose requires an accumulation of its reactive metabolite, NAPQI, after conjugation and

detoxification pathways become overwhelmed. The accumulated NAPQI is then able to complex with cellular proteins and macromolecules, precipitating downstream signaling events that lead to hepatocellular necrosis. Prior studies in humans, which demonstrated differences in liver toxicity after taking acetaminophen, indicated that the pharmacokinetics in humans were not associated with the toxicity response[52]. In accordance with the human data, our initial studies (*Aims 1* and *2*) that focused on determining biomarkers of sensitivity and response did not indicate that differential metabolism of acetaminophen contributed to the overall toxicity outcome. In *Aim* 3, pharmacokinetic profiling of key metabolites in five strains selected for differential susceptibility demonstrated that, while differences do exist among the inbred strains in the kinetics of acetaminophen metabolism, these differences do not completely explain the overall liver necrotic outcome. The outcome of this study was not surprising, given that acetaminophen-induced liver necrosis has a complex etiology; a variety of intra- and extra-cellular signaling cascades that are downstream of an apoptotic signaling cascade have been shown to modulate the necrotic outcome in the liver[56, 77].

The study demonstrated that, while metabolism is an important key event in the mode of action of acetaminophen toxicity, the overall necrotic outcome is not directly predicted by strain-dependent differences in its metabolism. The data emphasize that models such as the MMHP are a necessary tool for toxicologists seeking to determine novel genetic mediators that affect toxicity sensitivity.

B. STUDY CHALLENGES AND LIMITATIONS

Detection of additional genetic variation that may affect the acetaminophen liver toxicity outcome

A key feature of the MMHP as a research paradigm is the ability to model a large range of toxicity responses and to determine genetic loci predictive of a response based on the diversity across strains. Our work with acetaminophen builds support for the model as a means to determine susceptibility biomarkers that can translate to human populations. A limitation of the study to detect all possible acetaminophen-induced hepatotoxicity QTL is the use of a mouse diversity panel (MDP; *i.e.* commercially available inbred strains) as the basis for the MMHP. An MDP has many advantages to F2 populations that have been used traditionally for genetic mapping[155, 156] that include an increased phenotypic diversity, higher recombination frequencies, and the ability to acquire dense SNP maps that can be archived for each strain. However, the population structure among the strains of an MDP can complicate the analysis due to the semi-structured breeding programs from which they were derived. Common strain derivations over time have led to an over-representation of *Mus musculus domesticus* alleles and, thus, large regions of the genome are identical by descent across strains. Therefore, use of an MDP for genomic association studies can potentially lead to spurious associations to background genetic structure[157].

The best alternative to using an MDP population, the strains derived from the carefully controlled Collaborative Cross project[158, 159], are not yet available. The recently

developed Collaborative Cross mouse strains were designed specifically to incorporate large genetic variation[158]. To develop this resource, a controlled breeding program was designed to randomize genetic elements among the progeny derived from eight parental strains. A recent study demonstrated that the genetic variation present in the Collaborative Cross represents the optimal polymorphism architecture for the study of systems biology when compared to RI lines or to panels of classical inbred strains, and was demonstrated to be more reflective of the genetic variation expected in natural populations[74]. Use of the Collaborative Cross strains in future toxicogenetic mapping studies will improve the resolution of QTL detection by eliminating the genetic "blind spots" that occur within MDP strains[74].

Differences between mouse and human acetaminophen exposures

An additional limitation of our genetic biomarker studies is that the liver toxicity modeled in the MMHP is comparable to an acute overdose that may be different from the small ALT elevations observed in the human cohorts during a subchronic exposure at therapeutic doses. We make the assumption that there will be some commonalities in the mechanism of liver toxicity that occur in both exposure models because chemical dosing in human testing must be morally and ethically restrained. However, the discrepancy between the exposure conditions raises the possibility that there are genes

that control a propensity toward liver injury during a low dose, subchronic regimen that would be missed in a high dose exposure paradigm.

To correct this problem, we attempted to replicate the "human" low dose exposure regimen in a few mouse strains by administering a low dose of acetaminophen every 6 h to inbred mice in a subchronic regimen. However, we found that the mice, unlike humans, were resistant to ALT elevations at low doses, at least for the few strains tested (Appendix 6). We therefore hypothesized that the mice were protected against liver injury during a subchronic dosing regimen and concluded that perhaps liver protection was due, in part, to the healthy diet mice received, which is not representative of a typical human diet. One suggested approach to improving the low dose model in mice to achieve a similar degree of ALT elevations as humans would be to administer a high fat or "Western" diet to the mice that better reflects the typical human diet in the United States and that might predispose the mice toward liver injury. However, a study conducted by Ito *et al.* demonstrated that mice with steatotic livers achieved by administering a high fat and high carbohydrate diet were far less susceptible liver injury after an acute acetaminophen dose, owing to inhibition of CYP2E1 induction and a minimization of sinusoidal endothelial cell injury[160]. Therefore, it is likely that the low fat diet used was not protective against liver toxicity, but that instead the mice were simply adaptive to liver stress that may be caused by subchronic acetaminophen dosing. This conclusion is supported by a previous study that

demonstrated that mice given incrementally increasing doses of APAP for several days were subsequently protected against an otherwise lethal challenge dose[161].

Determination of early gene expression response biomarkers in mice and humans

A great promise of toxicogenomics is to determine biomarkers of effect that can add insight into the mechanism of toxicity and that can assess human exposure to environmental exposures and contaminants. In our microarray study, the phenotypic anchoring of the necrosis score to the transcript data enabled detection of gene response signatures. The study design was limited in its ability to fully characterize the mechanism of toxicity due to a lack of time course data. Because a single, late time point was examined (24 h) in which the maximum liver injury had already occurred, we were not able to examine early markers that might be predictive of later injury at a time when therapeutic intervention would be helpful. Acquisition of temporal information would provide for a basis to determine which gene changes initiate toxicity in the liver, potentially providing a basis for development of early therapeutic intervention in addition to the available antidote for acetaminophen poisoning, N-acetylcysteine.

Evidence of clinical or human *in vitro* translation of toxicogenomic biomarkers is essential in order to properly interpret the data. It is important to fully distinguish pathways that are associated with toxic effects from those pathways associated with a non-toxic physiological or pharmacological response. In examining datasets of sensitive

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"omics" data, it is also necessary to distinguish between changes that are adaptive in nature and those changes that are within a no observable adverse effect level (NOAEL). Determination of chemical NOAELs can only be accomplished by dose-response modeling of a variety of compounds in which a benchmark dose can be correlated with both transcript changes and *in vivo* pathology. Some progress has been made into developing computational tools that enable genomic benchmark dose analysis[162]. The challenge of determining the NOAEL becomes more complex as omics technologies become more refined, with greater throughput and sensitivity, and as data from multiple platforms becomes integrated. Therefore, translation between the transcriptional biomarkers collected from the mice given an overdose of acetaminophen with human data gathered after therapeutic doses will remain a challenge until a low dose mouse model of acetaminophen toxicity can be developed.

Collection of additional mouse acetaminophen metabolism endpoints

Complete validation of the model required an examination of whether strain-dependent differences in acetaminophen metabolism contributed to the overall toxicity response. Strain LP/J, the most resistant strain, demonstrated an increased serum exposure to the glucuronide and sulfate conjugates, which could potentially indicate an increased ability to clear parent compound via these pathways. However, there was also an increased serum exposure to the parent compound in LP/J mice, which

confounded interpretation of the data as far as assessing the potential for NAPQI-protein adduct formation. We next attempted to determine whether amounts of glutathione in liver could give insight into the detoxification of the reactive quinone radical[163]. However, because we measured GSH in livers at 6 h after treatment when GSH has been shown to be almost completely replenished, rather than at 2 h when the maximum depletion occurs[144], the significance of the data at 6 h must be questioned. It would have been beneficial in this study to examine GSH depletion in the context of temporal changes that might occur during the first four hours following overdose in order to determine whether resistant strains are deficient in the glutathione detoxification pathway. To measure hepatocellular GSH over time, livers of mice must be extracted at time points prior to 6 h. Additional liver samples were not available due to the study design which necessitated collecting plasma from each animal over time up to 6 h post-dosing.

C. FUTURE DIRECTIONS

Selection of candidate genes from genomic association analysis

An important challenge in the field of pharmacogenomics and in the use of the MMHP is the selection of the most likely or "right" candidates for follow-up evaluation and analysis using the most cost-efficient approach. Typically, this process entails computational approaches and database searching to predict which variants are most

likely to play a role in the response and that would have a high enough frequency of detection within the ethnic/racial groups studied. However, because such studies are resource-limited, potentially important sequence variants may be missed. Therefore, additional useful information may be yielded from sequencing polymorphisms within additional candidate genes in both the mouse and human cohorts to better define which genes are best associated with acetaminophen-induced liver toxicity. In our studies, potentially interesting candidates that were identified by haplotype-associated mapping include *Ptpn6* (*SHP-1*), a key signaling molecule in hematopoietic cells that is known to have many splice variants[164], and   *Prdm2*, a tumor suppressor gene that regulates heme oxygenase 1 (*Hmox1)* activity (Table 2.1).

Functional analysis of genetic variation within mouse Cd44

In order to better characterize the results of the genomic association study, further work needs to be done in order to elucidate the precise role that the *CD44* gene plays in propagating acetaminophen-induced liver injury. A careful approach needs to be considered due to the diverse roles of the CD44 protein in cell-cell interactions, cell adhesion and migration, and cellular growth and mitosis. It was recently noted that *Cd44* expression is repressed by *trp53* induction under stress in cell culture[165]. It may, therefore, be possible that functional variants are unable to efficiently respond to and repair necrotic injury within the liver, which allows an accumulation of signaling factors

and recruitment of an inflammatory response, leading to further cell damage. An important first step would be to measure *trp53* and *Cd44* expression in hepatocyte cell culture after acetaminophen administration in strains that express both alleles for the nonsynonymous coding SNP. If *Cd44* and *trp53* levels were found to differ by genotype, a potential follow-up analysis would involve measuring CD44-responsive cytokine and chemokine levels in resistant and susceptible mouse strains over a time course after acetaminophen treatment to determine whether differences in upstream *trp53* activation temporally affected downstream immune-mediated injury responses.

Analysis of toxicogenetic loci in the context of gene networks

The majority of pharmacogenetic traits that have been discovered to date have focused on a single gene and its role in affecting drug responses. It is more likely, however, that the genetic control of toxicity responses is governed by a network of genes as is observed in most complex diseases. Genome-wide association studies that focus on only the top several "most significant" SNPs may have some limitations that could be overcome by a pathway-based approach. These limitations are that: i) genes that contribute a smaller, but still significant, increase to disease risk may be overlooked, and ii) variants that confer a large effect may not be included if hundreds of thousands of markers have been tested and the sample size is relatively small. Wang *et al.* proposed a method in which the power to detect causal mechanisms of disease may be more

robust because multiple contributing factors are considered together, as opposed to focusing on a few SNPs with the highest association score[166]. This approach, which combines genome-wide association with the gene-set enrichment algorithm[167], identified a number of pathways that may be associated with Parkinson disease and age-related macular degeneration. In addition, the process of identifying candidate genes will be greatly simplified in the future with the creation of databases of "genetic" drug pathways, such as that proposed by the Pharmacogenetics and Pharmacogenomics Knowledge Database (www.pharmgkb.org). Such databases will facilitate the development of novel approaches to prioritize candidate genes in pharmaco- and toxicogenomic studies that would be useful in analyzing toxicity data collected from the MMHP.

Translation of mouse gene expression biomarkers to human data

A major limitation in the microarray study was an inability to compare liver transcript biomarkers of toxicity derived from the MMHP with gene expression data derived from humans, due to a lack of samples and a paucity of archived datasets comprising human liver microarray data with a similar acetaminophen overdose exposure. Translational analysis would also be greatly facilitated by repeating the study in mice using gene transcripts derived from a noninvasive tissue, such as circulating leukocytes in blood. Because these cells must be carefully collected and preserved to

extract RNA (such as in the specialized buffer supplied in Ambion RiboPure blood RNA isolation kits; Ambion, Austin, TX), the mouse exposures must be repeated in order to assay gene expression in blood. Once the data is collected from the MMHP, it would then be compared to human microarray data collected by our collaborators at UNC (Drs. Paul Watkins and Tong Zhou) that was derived from blood. Because this human data was derived from the study described in *Aim 1*, it would provide an ideal comparison to determine the utility of the MMHP approach for the discovery of liver injury biomarkers that may be used clinically.

Validation of the MMHP research paradigm with a pharmaceutical agent that causes idiosyncratic hepatotoxicity

Finally, a major challenge to the acceptance of the MMHP for pharmaceutical safety assessment is our selection of acetaminophen as a model toxicant to validate the approach as a method for uncovering rare adverse health events. Acetaminophen is considered a dose-dependent hepatotoxicant and not widely accepted to cause rare "idiosyncratic" liver injury, despite the data that demonstrates serum ALT elevations that occur during subchronic drug therapy in healthy adults[52]. In a retrospective analysis of nine acetaminophen clinical trials conducted by McNeil Consumer Healthcare (the manufacturer of Tylenol™) comprising 1039 patients, only 44 patients experienced an ALT elevation greater than 1.5 times the upper limit of normal while receiving the recommended dose of 3.9 g/day[79]. Of those patients that had subsequent ALT

measurements taken (33/41), 29 (93.5%) experienced either a decrease or complete resolution of serum ALT levels while on treatment[79]. While the resolution of ALT elevations during treatment has not been well studied, it can be inferred that there is an adaptive mechanism within the liver to prevent injury during low dose treatment. Perhaps owing to the liver's ability to adapt during treatment, acetaminophen has an excellent therapeutic safety profile.

Thus, it would be beneficial to validate the MMHP using a drug that clearly has idiosyncratic properties in which observed toxicity is not correlative with dose. However, due to the rarity of these events, it is unlikely that toxicity could be detected using a conventional exposure, even within a diverse panel of strains. Mouse models of idiosyncratic liver toxicity have been developed that capitalize on the observation that an episode of inflammatory stress can lower the threshold for which a susceptible individual will experience toxicity. Animal models have been developed in which lipopolysaccharide (LPS) treatment has rendered sensitivity to drugs that cause idiosyncratic liver toxicity, such as diclofenac[168], chlorpromazine[169], and trovafloxacin[170]. It may therefore be beneficial to combine the LPS exposure paradigm with the diversity found within the MMHP in order to determine the genetic basis underlying susceptibility to idiosyncratic drugs for which genetic screening may improve the safety profile.

D. SUMMARY

Collectively, our results indicate that the Mouse Model of the Human Population is a valuable tool for the development of biomarkers that enable pre-screening of patients prior to dosing with pharmaceuticals that have potentially toxic side effects. The model, used in conjunction with toxicogenomic approaches, identified CD44 as a protein that may be involved in modulating sensitivity to acetaminophen-induced liver injury. Furthermore, the model has utility for determining transcript biomarkers of response that can aid researchers in both risk assessment and the study of toxicity mechanisms. In addition, the toxicity outcome could not be predicted based on strain-specific metabolic profiles, further emphasizing the need for toxicogenomic approaches to be used that can better predict susceptibility factors. The data acquired with the MMHP could therefore be influential in the analysis of individual risk to pharmaceutical or environmental agents and may facilitate both drug development and human risk assessment.

Toxicogenomics and toxicogenetics approaches provide a means to enable the linking of traditional toxicological endpoints with novel molecular targets that are mechanistically involved in adverse responses. While toxicogenomics is unlikely to replace classical toxicological testing paradigms, it enriches the field by allowing researchers to discover biomarkers and subtle changes that occur sub-clinically or that predispose susceptible individuals toward an injurious event. By using a carefully

designed rodent model that is designed to capture the range of genetic variation, toxicologists will be better able inform risk assessment for human populations. The field will continue to expand in the coming years now that the Food and Drug Administration (FDA), along with the Environmental Protection Agency (EPA) are accepting submissions of functional polymorphism and microarray data as part of the regulatory process[3]. The successful implementation of rodent models to this field will require a careful study design with special attention for the selection of appropriate strains and relevant time points. Translational research bridging rodent and human toxicity will be the key to the success of using toxicogenomics to facilitate "personalized" medicine. With additional validation, translational toxicogenomic approaches that utilize a genetically heterogeneous rodent model, such as the MMHP, will revolutionize the field of toxicology.

APPENDICES

Appendix 1

Primers used for genetic sequence analysis in mice and humans

Gene	Species	Upstream Primer	Downstream Primer
Cd44	Mouse	TCCTTCTCCGTCATTTCCAC	TGTGGGGTCTCCTCTTCATC
Capn8	Mouse	CTGAGGCCATGGTAGCATTT	CATAAGACGGGACCCTTGAA
Ly86	Mouse	GCCGTTGAGCCTTGAGTTAC	CATTCAGGAAAAAGCCTCCA
Cd59a	Mouse	AGGGTTGAAGTAGGGGAGGA	CAGCTACATTGCAGGAACCA
Cat	Mouse	GTGGGGGTGTCCTCTAGTGA	AACCACAAAACCGGAAACAA
Cyp2e1	Mouse	CCTGTAAAGGGAGACCCACA	AAGGGGACAAGGCTCTCATT
CD44	Human	CCTCTTGGCCAGATGTGAAT	AAGCCACATAGCACCATTCC
Capn10	Human	AAAGCCCCTGATGATGTGAC	GGCGAGCACTAAGACTCCAG
Ly86	Human	CCAATATTTGTGGCATGAATGA	GCCAAAATGACAAAGCCAGT
CD59	Human	GCCTTACACTAGCCCACCTG	AAGTTTTGGGGGAGTCAAAA

Appendix 2

Human subject genotypes for SNPs within *CD44*, *CD59*, *CAPN10*, and *LY86*

Subject	Study	Treatment	Gender	CD44	CD59	CAPN10	LY86
1	UNC	APAP	Female	C/C	AT/-	G/G	AGG/AGG
3	UNC	APAP	Male	C/C	AT/AT	G/A	AGG/AGG
4	UNC	APAP	Male	C/C	AT/-	A/A	AGG/-
5	UNC	APAP	Male	C/C	AT/AT	G/A	AGG/AGG
6	UNC	APAP	Male	C/C	AT/-	A/A	AGG/-
7	UNC	APAP	Male	C/C	AT/-	G/A	AGG/-
8	UNC	APAP	Female	C/C	AT/AT	G/G	AGG/AGG
10	UNC	APAP	Female	C/C	AT/AT	A/A	AGG/-
11	UNC	APAP	Male	C/C	AT/-	G/A	AGG/-
14	UNC	APAP	Female	C/T	AT/-	A/A	AGG/AGG
15	UNC	APAP	Male	C/C	-/-	G/G	AGG/-
16	UNC	APAP	Female	C/C	AT/AT	G/A	AGG/-
17	UNC	APAP	Male	C/C	-/-	A/A	AGG/AGG
19	UNC	APAP	Female	C/C	AT/AT	A/A	AGG/AGG
20	UNC	APAP	Female	C/C	AT/AT	A/A	AGG/-
21	UNC	APAP	Male	C/C	AT/-	G/A	-/-
22	UNC	APAP	Female	C/C	AT/AT	G/A	AGG/-
23	UNC	APAP	Male	C/C	AT/AT	G/G	AGG/AGG
24	UNC	APAP	Male	C/C	AT/-	A/A	AGG/AGG
26	UNC	APAP	Male	C/T	AT/AT	A/A	AGG/AGG
27	UNC	APAP	Female	C/C	AT/-	G/A	AGG/-
28	UNC	APAP	Female	C/C	AT/AT	G/G	AGG/AGG
29	UNC	APAP	Female	C/C	AT/AT	G/G	AGG/AGG
30	UNC	APAP	Female	C/C	AT/-	A/A	AGG/AGG
32	UNC	APAP	Male	C/C	AT/-	A/A	AGG/AGG
33	UNC	APAP	Male	C/C	AT/AT	G/G	AGG/AGG
34	UNC	APAP	Male	C/C	AT/-	G/G	AGG/AGG
37	UNC	APAP	Male	C/C	AT/AT	G/G	AGG/AGG
39	UNC	APAP	Female	C/C	AT/AT	G/G	AGG/AGG
40	UNC	APAP	Female	C/C	AT/AT	G/A	AGG/AGG
41	UNC	APAP	Male	C/C	AT/-	G/G	AGG/AGG
42	UNC	APAP	Male	C/T	-/-	A/A	AGG/-
43	UNC	APAP	Male	C/T	AT/AT	G/A	AGG/AGG
44	UNC	APAP	Female	C/C	AT/-	G/G	AGG/AGG
45	UNC	APAP	Male	C/C	AT/AT	A/A	AGG/-
46	UNC	APAP	Female	C/C	AT/AT	G/G	AGG/-
47	UNC	APAP	Male	C/C	AT/AT	G/A	AGG/AGG
48	UNC	APAP	Male	C/C	AT/AT	G/G	AGG/AGG
49	UNC	APAP	Female	C/C	AT/-	G/A	AGG/AGG
50	UNC	APAP	Male	C/C	AT/-	G/A	AGG/AGG
53	UNC	APAP	Female	C/C	AT/-	G/A	AGG/-
54	UNC	APAP	Male	C/C	AT/AT	A/A	AGG/AGG

56	UNC	APAP	Male	C/T	AT/AT	A/A	AGG/AGG
57	UNC	APAP	Male	C/T	AT/-	A/A	AGG/AGG
58	UNC	APAP	Female	C/C	AT/AT	G/G	AGG/AGG
62	UNC	APAP	Female	C/C	AT/AT	G/A	AGG/AGG
63	UNC	APAP	Male	C/C	AT/AT	G/G	AGG/AGG
64	UNC	APAP	Male	C/C	AT/AT	G/A	AGG/AGG
65	UNC	APAP	Male	C/C	AT/-	G/G	AGG/AGG
1025	Purdue Pharma	Morphine +APAP	Female	C/C	-/-	G/A	AGG/-
1030	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/-	G/A	AGG/-
1034	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/AT	G/A	AGG/AGG
1036	Purdue Pharma	Morphine +APAP	Male	C/C	AT/-	A/A	AGG/AGG
1040	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/-	G/G	AGG/AGG
1042	Purdue Pharma	Morphine +APAP	Female	C/C	AT/-	A/A	AGG/-
1046	Purdue Pharma	APAP	Male	C/C	AT/-	G/A	AGG/AGG
1048	Purdue Pharma	APAP	Male	C/T	AT/AT	A/A	AGG/-
1051	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/-	G/G	AGG/AGG
1058	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/-	G/A	AGG/AGG
1059	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/AT	A/A	AGG/-
1070	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/-	G/A	AGG/-
1072	Purdue Pharma	Hydromorphone +APAP	Male	C/C	-/-	G/G	AGG/AGG
1074	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/-	A/A	AGG/-
1075	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/-	G/A	-/-
1076	Purdue Pharma	APAP	Male	C/C	AT/AT	G/G	AGG/AGG
1079	Purdue Pharma	Morphine +APAP	Male	C/C	AT/-	A/A	-/-
1080	Purdue Pharma	Hydromorphone +APAP	Female	C/C	AT/AT	G/G	AGG/AGG
1081	Purdue Pharma	APAP	Male	C/T	AT/AT	G/G	AGG/-
1096	Purdue Pharma	APAP	Male	C/C	AT/AT	G/G	AGG/AGG
1105	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	G/A	AGG/-
1109	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/AT	A/A	AGG/-
1110	Purdue Pharma	Morphine +APAP	Male	C/C	AT/-	G/G	AGG/AGG
1116	Purdue	Hydromorphone +APAP	Male	C/T	AT/-	G/A	AGG/-

	Pharma						
1119	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	G/A	AGG/AGG
1120	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	G/G	AGG/AGG
1123	Purdue Pharma	APAP	Male	C/C	AT/-	A/A	AGG/-
1127	Purdue Pharma	APAP	Female	C/C	AT/AT	A/A	AGG/-
1129	Purdue Pharma	Morphine +APAP	Male	C/C	AT/-	G/G	AGG/-
1131	Purdue Pharma	Morphine +APAP	Male	C/T	AT/AT	G/A	AGG/AGG
1132	Purdue Pharma	Oxycodone +APAP	Male	C/C	-/-	G/G	AGG/AGG
1137	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	G/G	AGG/AGG
1138	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/-	G/A	AGG/-
1142	Purdue Pharma	Oxycodone +APAP	Male	C/T	AT/AT	A/A	AGG/AGG
1146	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	G/A	AGG/AGG
1147	Purdue Pharma	APAP	Male	C/C	-/-	G/A	AGG/-
1150	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/-	G/G	AGG/AGG
1158	Purdue Pharma	APAP	Female	C/C	AT/-	G/G	AGG/-
1164	Purdue Pharma	Hydromorphone +APAP	Female	C/C	AT/AT	G/A	AGG/-
1165	Purdue Pharma	Morphine +APAP	Male	C/C	AT/AT	G/A	AGG/-
1167	Purdue Pharma	APAP	Male	C/T	AT/AT	A/A	AGG/-
1170	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/AT	A/A	AGG/-
1177	Purdue Pharma	APAP	Male	C/T	AT/-	A/A	AGG/AGG
1178	Purdue Pharma	Oxycodone +APAP	Female	C/C	AT/-	G/A	AGG/AGG
1180	Purdue Pharma	Morphine +APAP	Male	C/C	AT/AT	A/A	AGG/-
1181	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	A/A	AGG/AGG
1185	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/-	G/A	AGG/AGG
1188	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	G/G	AGG/AGG
1196	Purdue Pharma	Morphine +APAP	Male	C/T	AT/-	G/A	AGG/AGG
1203	Purdue Pharma	Oxycodone +APAP	Female	C/C	AT/AT	G/A	AGG/AGG
1204	Purdue Pharma	Morphine +APAP	Male	C/C	AT/AT	G/A	AGG/AGG

1206	Purdue Pharma	Morphine +APAP	Male	C/C	AT/-	G/G	AGG/AGG
1217	Purdue Pharma	APAP	Female	C/C	AT/AT	G/G	AGG/AGG
1218	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/AT	A/A	AGG/-
1219	Purdue Pharma	APAP	Female	C/C	AT/AT	G/A	AGG/-
1226	Purdue Pharma	Hydromorphone +APAP	Female	C/C	AT/AT	G/A	AGG/-
1228	Purdue Pharma	Morphine +APAP	Female	C/T	AT/AT	G/A	AGG/AGG
1230	Purdue Pharma	Morphine +APAP	Female	C/C	AT/-	G/A	AGG/AGG
2009	Purdue Pharma	APAP	Male	C/C	AT/AT	A/A	-/-
2013	Purdue Pharma	Morphine +APAP	Male	C/C	AT/AT	G/A	AGG/-
2014	Purdue Pharma	Morphine +APAP	Female	C/C	-/-	G/G	AGG/AGG
2019	Purdue Pharma	APAP	Male	C/T	AT/-	G/A	AGG/-
2026	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	G/A	-/-
2032	Purdue Pharma	APAP	Male	C/C	-/-	G/A	AGG/AGG
2033	Purdue Pharma	Oxycodone +APAP	Male	C/C	AT/AT	G/A	AGG/AGG
2046	Purdue Pharma	Morphine +APAP	Female	C/C	-/-	G/A	-/-
2060	Purdue Pharma	APAP	Female	T/T	AT/-	G/A	AGG/AGG
2068	Purdue Pharma	Oxycodone +APAP	Female	C/T	AT/AT	A/A	-/-
2070	Purdue Pharma	Hydromorphone +APAP	Male	C/C	-/-	A/A	-/-
2079	Purdue Pharma	APAP	Female	C/C	AT/AT	A/A	ND
2080	Purdue Pharma	Hydromorphone +APAP	Female	C/T	AT/AT	A/A	AGG/AGG
2081	Purdue Pharma	Oxycodone +APAP	Male	C/T	AT/-	G/G	AGG/-
2082	Purdue Pharma	Oxycodone +APAP	Male	C/T	AT/AT	A/A	-/-
2088	Purdue Pharma	Morphine +APAP	Female	C/C	AT/-	A/A	-/-
2105	Purdue Pharma	APAP	Male	C/C	AT/-	G/A	AGG/AGG
2110	Purdue Pharma	Hydromorphone +APAP	Male	C/C	AT/AT	G/G	-/-

Appendix 3

Serum ALT information from human volunteers in the UNC and Purdue Pharma cohorts used for correlation analysis with subject genotype

Subject	Study	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	UNC	22	23	19	26	21	26	22
3	UNC	21	23	22	29	31	31	34
4	UNC	19	14	24	20	24	31	31
5	UNC	23	28	37	56	83	88	88
6	UNC	24	27	25	23	26	38	39
7	UNC	20	19	17	20	33	22	24
8	UNC	23	23	23	29	30	29	34
10	UNC	29	33	29	33	43	52	59
11	UNC	88	100	154	290	415	460	380
14	UNC	23	25	29	23	33	30	36
15	UNC	31	25	25	29	31	38	48
16	UNC	34	31	30	42	66	84	108
17	UNC	23	30	23	24	24	30	31
19	UNC	63	64	64	61	76	65	66
20	UNC	18	15	22	29	44	57	78
21	UNC	31	40	43	49	50	55	56
22	UNC	36	36	40	41	46	52	58
23	UNC	31	32	35	50	69	74	79
24	UNC	31	29	35	41	47	53	56
26	UNC	34	36	35	39	44	48	49
27	UNC	24	26	26	28	34	42	47
28	UNC	28	31	33	53	68	72	63
29	UNC	29	24	27	29	33	32	37
30	UNC	30	36	33	34	38	40	38
32	UNC	56	58	58	58	62	65	67
33	UNC	56	51	52	56	62	63	63
34	UNC	32	35	40	46	49	53	57
37	UNC	34	37	34	36	46	52	57
39	UNC	25	30	29	34	47	46	43
40	UNC	26	23	26	28	32	32	36
41	UNC	36	43	36	59	100	188	218
42	UNC	29	30	28	31	32	29	25
43	UNC	40	44	44	53	75	83	84
44	UNC	32	31	29	28	30	31	32
45	UNC	37	31	36	36	41	38	42
46	UNC	31	38	49	59	73	84	113
47	UNC	40	41	38	35	41	40	45
48	UNC	31	30	31	27	25	32	32
49	UNC	31	33	32	32	36	36	36
50	UNC	24	31	33	34	33	30	33
53	UNC	27	32	38	36	43	51	60

54	UNC	37	39	39	38	39	41	42
56	UNC	43	44	48	56	80	102	135
57	UNC	36	35	39	75	116	166	174
58	UNC	23	22	21	26	24	25	26
62	UNC	71	68	69	71	76	94	97
63	UNC	24	28	31	51	71	83	95
64	UNC	53	55	55	54	60	66	68
65	UNC	48	51	53	57	56	54	54
1025	Purdue Pharma	11	27	74	56	87	106	94
1030	Purdue Pharma	14	17	21	34	49	55	72
1034	Purdue Pharma	10	11	14	33	114	284	328
1036	Purdue Pharma	26	29	38	120	123	150	202
1040	Purdue Pharma	15	12	13	18	34	76	140
1042	Purdue Pharma	9	10	9	10	16	21	22
1046	Purdue Pharma	21	20	22	26	32	29	37
1048	Purdue Pharma	46	66	91	133	211	233	246
1051	Purdue Pharma	12	12	12	13	13	13	14
1058	Purdue Pharma	21	22	26	36	42	47	57
1059	Purdue Pharma	20	23	33	69	121	149	196
1070	Purdue Pharma	16	13	19	31	41	55	70
1072	Purdue Pharma	15	16	17	27	38	56	86
1074	Purdue Pharma	23	24	32	49	55	73	92
1075	Purdue Pharma	14	49	48	61	94	79	99
1076	Purdue Pharma	16	25	44	90	127	146	214
1079	Purdue Pharma	12	20	25	23	22	25	24
1080	Purdue Pharma	14	12	12	12	13	14	15
1081	Purdue Pharma	30	30	39	67	88	93	109
1096	Purdue Pharma	38	34	38	59	113	146	159
1105	Purdue Pharma	18	18	34	44	70	115	159
1109	Purdue Pharma	20	22	20	24	28	33	38
1110	Purdue Pharma	26	23	26	38	45	58	72
1116	Purdue Pharma	34	70	97	132	281	321	281
1119	Purdue Pharma	16	16	19	34	83	171	307
1120	Purdue Pharma	24	23	29	47	59	63	59
1123	Purdue Pharma	17	28	19	28	30	33	39
1127	Purdue Pharma	9	10	11	19	31	34	34
1129	Purdue Pharma	13	11	13	21	25	26	61
1131	Purdue Pharma	13	14	11	16	22	34	42
1132	Purdue Pharma	18	18	19	21			
1137	Purdue Pharma	18	19	19	20	24	21	19
1138	Purdue Pharma	16	14	30	65	74	100	69
1142	Purdue Pharma	24	25	24	23		26	27
1146	Purdue Pharma	28	25	33	56	67	65	
1147	Purdue Pharma	17	18	19	33	69	66	61
1150	Purdue Pharma	7	9	9	9	7	10	9
1158	Purdue Pharma	18	17	21	21	16	19	16
1164	Purdue Pharma	29	32	42	86	117	146	165
1165	Purdue Pharma	11	11	15	17	17	26	30

1167	Purdue Pharma	44	41	43	60	70	82	75
1170	Purdue Pharma	10	8	11	9	8	12	10
1177	Purdue Pharma	35	33	32	48	66	103	91
1178	Purdue Pharma	26	25	30	49	61	65	105
1180	Purdue Pharma	17	42	107		122	156	192
1181	Purdue Pharma	14	12	15	21	24	26	24
1185	Purdue Pharma	25	21	28	33	33	40	45
1188	Purdue Pharma	16	17	24	29	27	37	38
1196	Purdue Pharma	40	95	174	163	250	223	166
1203	Purdue Pharma	10	11	13	18	22	41	62
1204	Purdue Pharma	12	15	15	17	25	42	47
1206	Purdue Pharma	13	13	15	27	29	39	47
1217	Purdue Pharma	25	26	30	39	56	94	79
1218	Purdue Pharma	23	29	37	34	31	32	35
1219	Purdue Pharma	9	12	19	35	66	80	76
1226	Purdue Pharma	12	11	14	23	25	33	45
1228	Purdue Pharma	16	29	178	400	459	403	316
1230	Purdue Pharma	11	120	260	205	205	343	321
2009	Purdue Pharma	12	13	13	17	22	27	26
2013	Purdue Pharma	28	22	25	38	33	31	32
2014	Purdue Pharma	10	10	11	13	14	14	13
2019	Purdue Pharma	38	38	35	35	37	48	55
2026	Purdue Pharma	26	27	36	46	63	90	125
2032	Purdue Pharma	8	10	12	23	46	58	71
2033	Purdue Pharma	25	38	27	36	68	107	138
2046	Purdue Pharma	8	9	12	13	17	17	19
2060	Purdue Pharma	9	9	11	11	13	14	19
2068	Purdue Pharma	13	11	16	18	19	23	39
2070	Purdue Pharma	12	27	46	91	400	258	206
2079	Purdue Pharma	6	6	6	7	12	14	18
2080	Purdue Pharma	11	10	10	11	11	9	12
2081	Purdue Pharma	17	17	21	90	70	56	60
2082	Purdue Pharma	17	16	17	20	32	41	59
2088	Purdue Pharma	6	10	20	22	17	16	16
2105	Purdue Pharma	10	12	11	13	16	15	21
2110	Purdue Pharma	17	17	39	121	194	249	290

Appendix 4

Daily ALT values for subjects enrolled in the UNC acetaminophen trial

Subject	Day -14	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
1	21	25	19	16	17	22	23	19	26	21	26	22
3	14	19	20	20	19	21	23	22	29	31	31	34
4	21	20	23	22	21	19	14	24	20	24	31	31
5	35	29	29	24	24	23	28	37	56	83	88	88
6	33	30	32	29	31	24	27	25	23	26	38	39
7	23	15	17	19	17	20	19	17	20	33	22	24
8	21	13	15	15	23	23	23	23	29	30	29	34
10	24	25	27	33	29	29	33	29	33	43	52	59
11	63	71	73	90	96	88	100	154	290	415	460	380
14	19	26	26	31	31	31	25	25	29	31	38	48
15	22	34	36	27	34	34	31	30	42	66	84	108
16	44	26	21	25	25	23	30	23	24	24	30	31
17	26	48	50	75	75	63	64	64	61	76	65	66
19	41	19	19	19	24	18	15	22	29	44	57	78
20	20	31	33	35	33	31	40	43	49	50	55	56
21	39	38	33	34	38	36	36	40	41	46	52	58
22	41	29	30	30	31	31	32	35	50	69	74	79
23	28	29	28	33	35	31	29	35	41	47	53	56
24	33	24	26	24	24	24	26	26	28	34	42	47
26	34	27	26	23	33	28	31	33	53	68	72	63
27	32	22	17	21	27	29	24	27	29	33	32	37
28	29	29	30	32	35	30	36	33	34	38	40	38
29	20	52	57	52	58	56	58	58	58	62	65	67
30	32	61	60	60	67	56	51	52	56	62	63	63
32	64	33	38	35	39	32	35	40	46	49	53	57
33	70	41	41	46	43	34	37	34	36	46	52	57
34	30	31	27	29	30	25	30	29	34	47	46	43
37	63	21	23	25	29	26	23	26	28	32	32	36
39	28	33	40	38	40	36	43	36	59	100	188	218
40	ND	30	31	31	35	32	31	29	28	30	31	32
41	31	35	39	42	37	37	31	36	36	41	38	42
42	29	29	29	32	31	31	38	49	59	73	84	113
43	31	33	35	39	37	40	41	38	35	41	40	45
44	27	32	30	30	35	31	30	31	27	25	32	32
45	41	33	33	27	33	31	33	32	32	36	36	36
46	33	28	20	27	28	24	31	33	34	33	30	33
47	37	26	25	24	25	27	32	38	36	43	51	60
48	30	33	33	35	37	37	39	39	38	39	41	42
49	37	23	22	21	23	23	22	21	26	24	25	26
50	30	54	58	66	83	71	68	69	71	76	94	97
53	35	35	34	32	33	24	28	31	51	71	83	95
54	43	47	47	55	53	53	55	55	54	60	66	68

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56	52	32	34	39	48	48	51	53	57	56	54	54
57	23	27	26	26	26	23	25	29	23	33	30	36
58	31	37	32	33	32	34	36	35	39	44	48	49
62	50	30	33	30	30	29	30	28	31	32	29	25
63	25	41	37	36	40	40	44	44	53	75	83	84
64	49	42	45	42	46	43	44	48	56	80	102	135
65	42	29	25	35	40	36	35	39	75	116	166	174

Subject	Day 11	Day 12	Day 13	Day 14	Day 28
1	22	18	20	19	24
3	34	38	40	40	16
4	31	35	38	48	35
5	88	76	80	77	39
6	39	50	59	57	30
7	24	25	27	26	25
8	34	36	ND	31	20
10	59	64	63	62	30
11	380	314	256	217	63
14	48	47	52	47	41
15	108	119	116	133	23
16	31	39	37	35	56
17	66	52	46	44	29
19	78	85	95	102	53
20	56	52	50	50	25
21	58	57	63	58	36
22	79	74	68	65	31
23	56	52	55	52	27
24	47	49	49	49	33
26	63	57	50	74	33
27	37	31	31	31	31
28	38	38	33	31	33
29	67	77	76	79	21
30	63	66	68	69	35
32	57	53	51	48	57
33	57	52	49	52	54
34	43	43	ND	49	47
37	36	35	36	38	42
39	218	206	226	225	ND
40	32	36	41	48	28
41	42	42	45	42	43
42	113	108	89	91	32
43	45	41	48	48	31
44	32	40	36	37	30
45	36	38	38	43	47
46	33	36	28	28	30
47	60	51	60	60	35
48	42	38	36	40	27

49	26	27	31	31	36
50	97	98	103	116	23
53	95	102	95	77	31
54	68	71	85	86	46
56	54	60	64	67	79
57	36	37	37	48	50
58	49	47	46	ND	34
62	25	30	36	39	84
63	84	89	86	81	31
64	135	184	220	231	59
65	174	210	192	165	30

Appendix 5

Linear regression analysis of human serum ALT and α-GST levels[2]

Linear regression analysis and descriptive statistics of the bivariate fit of serum enzyme levels. Plotted parameters are the natural log of the maximum levels of ALT and α-GST achieved during treatment. Levels were measured in subjects within the Purdue Pharma cohort that were administered the maximum recommended dose of acetaminophen (4 g every 6 h for 14 days)[52]. The green line represents the linear regression line and the red line represents the fitted mean of the data.
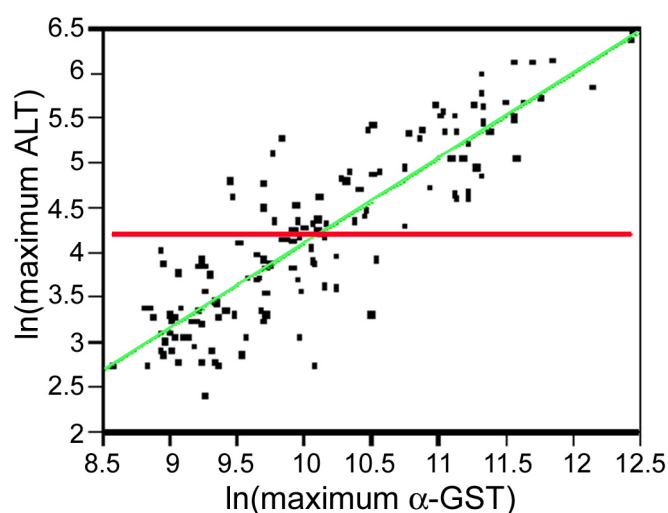
Linear Fit

ln(maximum ALT) = -5.386887 + 0.948964 ln(maximum α-GST)

Summary of Fit

R^2	0.762295
R^2 Adj	0.760633
Root Mean Square Error	0.472801
Mean of Response	4.201007
Observations (or Sum Weights)	145

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	102.51275	102.513	458.5861
Error	143	31.96635	0.224	Prob > F
C. Total	144	134.47910		<.0001

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Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-5.386887	0.449445	-11.99	<.0001
lnMax_GST	0.948964	0.044314	21.41	<.0001

Appendix 6

Liver injury in B6C3F1/J and DBA/2J mice following a low dose subchronic acetaminophen exposure

Serum ALT levels were measured daily from mice treated with 50 mg/kg (A; strain B6C3F1/J) or 100 mg/kg (B; strain DBA/2J) per day of acetaminophen in four divided doses (one dose every 6 h, *i.g.* for a total dosing volume of 10 ml/kg). Baseline ALT quantifications were conducted on days 1-3 of the study. Dosing began on the morning of day 4 and ended on the morning of day 11. Lines represent values for individual animals. In panel B, naïve animals are represented in green, vehicle controls are represented in blue, and APAP-treated animals are represented in red.
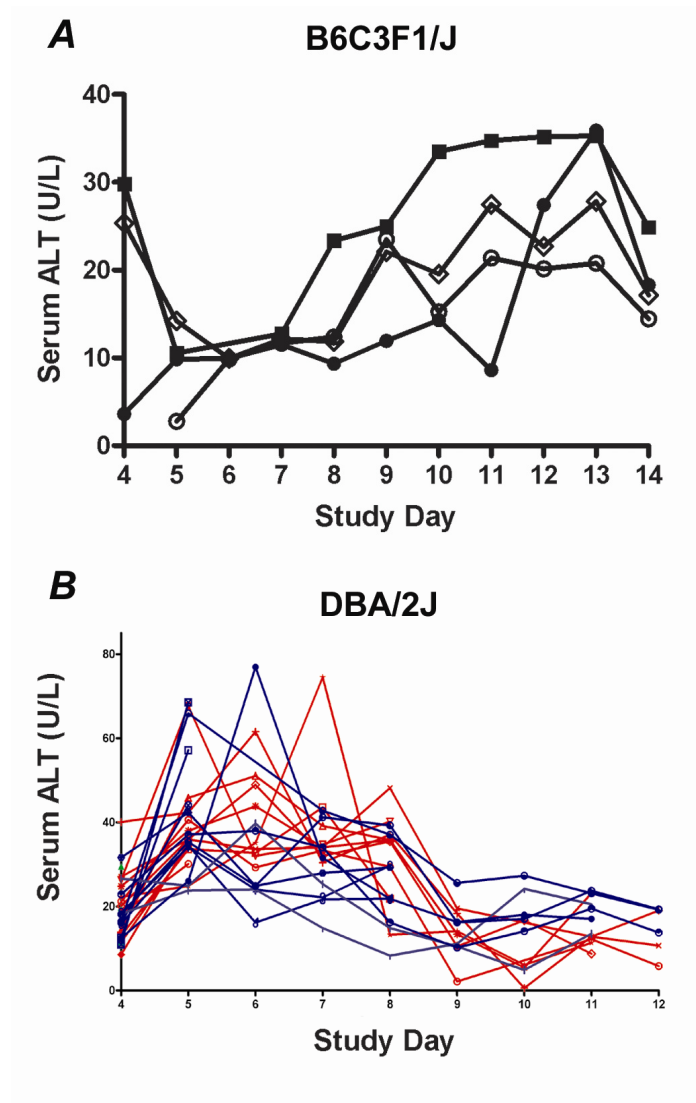
REFERENCES

Reference List

1. …..Gutman,S. & Hackett,J. Search for shortcuts on the critical path to market: US FDA perspectives from the diagnostic side. *Pharmacogenomics*. **7**, 1223-1227 (2006).

2. …..U.S.Food and Drug Administration. Guidance for Industry: Pharmacogenomic Data Submissions. 2005.

3. …..Frueh,F.W. Impact of microarray data quality on genomic data submissions to the FDA. *Nat Biotechnol*. **24**, 1105-1107 (2006).

4. …..Bammler,T., Beyer,R.P., Bhattacharya,S., Boorman,G.A., Boyles,A. et al. Standardizing global gene expression analysis between laboratories and across platforms. *Nat. Methods* **2**, 351-356 (2005).

5. …..Shi,L., Reid,L.H., Jones,W.D., Shippy,R., Warrington,J.A. et al. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol*. **24**, 1151-1161 (2006).

6. …..Beyer,R.P., Fry,R.C., Lasarev,M.R., McConnachie,L.A., Meira,L.B. et al. Multicenter study of acetaminophen hepatotoxicity reveals the importance of biological endpoints in genomic analyses. *Toxicol Sci* **99**, 326-337 (2007).

7. …..Ishkanian,A.S., Malloff,C.A., Watson,S.K., DeLeeuw,R.J., Chi,B. et al. A tiling resolution DNA microarray with complete coverage of the human genome. *Nat Genet* **36**, 299-303 (2004).

8. …..Arcellana-Panlilio,M. & Robbins,S.M. Cutting-edge technology. I. Global gene expression profiling using DNA microarrays. *Am J Physiol Gastrointest. Liver Physiol* **282**, G397-G402 (2002).

9. …..Paules,R. Phenotypic anchoring: Linking cause and effect. *Environ. Health Perspect.* **111**, A338-A339 (2003).

10. …..Powell,C.L., Kosyk,O., Ross,P.K., Schoonhoven,R., Boysen,G. et al. Phenotypic anchoring of acetaminophen-induced oxidative stress with gene expression profiles in rat liver. *Toxicol. Sci* **93**, 213-222 (2006).

11. . …Heinloth,A.N., Irwin,R.D., Boorman,G.A., Nettesheim,P., Fannin,R.D. et al. Gene expression profiling of rat livers reveals indicators of potential adverse effects. *Toxicol. Sci*. **80**, 193-202 (2004).

12. .….Wang,E.J., Snyder,R.D., Fielden,M.R., Smith,R.J., & Gu,Y.Z. Validation of putative genomic biomarkers of nephrotoxicity in rats. *Toxicology* **246**, 91-100 (2008).

13. .….Khor,T.O., Ibrahim,S., & Kong,A.N. Toxicogenomics in drug discovery and drug development: potential applications and future challenges. *Pharm Res* **23**, 1659-1664 (2006).

14. .….Ridd,K., Zhang,S.D., Edwards,R.E., Davies,R., Greaves,P. et al. Association of gene expression with sequential proliferation, differentiation and tumor formation in murine skin. *Carcinogenesis* **27**, 1556-1566 (2006).

15. ….Christians,U., Schmitz,V., Schoning,W., Bendrick-Peart,J., Klawitter,J. et al. Toxicodynamic therapeutic drug monitoring of immunosuppressants: promises, reality, and challenges. *Ther Drug Monit*. **30**, 151-158 (2008).

16. ....Bushel,P.R., Heinloth,A.N., Li,J., Huang,L., Chou,J.W. et al. Blood gene expression signatures predict exposure levels. *Proc. Natl. Acad Sci U. S. A* **104**, 18211-18216 (2007).

17. ...National Research Council Toxicity Testing in the 21st Century: A Vision and a Strategy(National Academies Press, Washington, DC, 2007).

18. ....Zeeberg,B.R., Feng,W., Wang,G., Wang,M.D., Fojo,A.T. et al. GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol* **4**, R28 (2003).

19. ....Barry,W.T., Nobel,A.B., & Wright,F.A. Significance analysis of functional categories in gene expression studies: a structured permutation approach. *Bioinformatics* **21**, 1943-1949 (2005).

20. ....Khatri,P., Bhavsar,P., Bawa,G., & Draghici,S. Onto-Tools: an ensemble of

web-accessible, ontology-based tools for the functional design and interpretation of high-throughput gene expression experiments. *Nucleic Acids Res* **32**, W449-W456 (2004).

21. ....Burns,J.J. IV. Pharmacogenetics and drug toxicity. Variation of drug metabolism in animals and the prediction of drug action in man. *Ann N Y. Acad Sci* **151**, 959-967 (1968).

22. ....Kalow,W. Contribution of hereditary factors to the response to drugs. *Fed. Proc.* **24**, 1259-1265 (1965).

23. ....Vesell,E.S. & Page,J.G. Genetic control of dicumarol levels in man. *J Clin Invest* **47**, 2657-2663 (1968).

24. ....Nebert,D.W., Zhang,G., & Vesell,E.S. From human genetics and genomics to pharmacogenetics and pharmacogenomics: past lessons, future directions. *Drug Metab Rev* **40**, 187-224 (2008).

25. ....Cacabelos,R. Pharmacogenetic basis for therapeutic optimization in Alzheimer's disease. *Mol Diagn. Ther* **11**, 385-405 (2007).

26. ....Yates,C.R., Krynetski,E.Y., Loennechen,T., Fessing,M.Y., Tai,H.L. et al. Molecular diagnosis of thiopurine S-methyltransferase deficiency: genetic basis for azathioprine and mercaptopurine intolerance. *Ann Intern. Med* **126**, 608-614 (1997).

27. ....Relling,M.V., Hancock,M.L., Rivera,G.K., Sandlund,J.T., Ribeiro,R.C. et al. Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. *J Natl. Cancer Inst.* **91**, 2001-2008 (1999).

28. ....Rost,S., Fregin,A., Ivaskevicius,V., Conzelmann,E., Hortnagel,K. et al. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* **427**, 537-541 (2004).

29. ....Higashi,M.K., Veenstra,D.L., Kondo,L.M., Wittkowsky,A.K., Srinouanprachanh,S.L. et al. Association between CYP2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. *J. Am. Med. Assoc.* **287**, 1690-1698 (2002).

30. ....Rieder,M.J., Reiner,A.P., Gage,B.F., Nickerson,D.A., Eby,C.S. et al. Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med* **352**, 2285-2293 (2005).

31. ....Weinshilboum,R.M. & Wang,L. Pharmacogenetics and pharmacogenomics: development, science, and translation. *Annu Rev Genomics Hum. Genet* **7**, 223-245 (2006).

32. ....Venter,J.C., Adams,M.D., Myers,E.W., Li,P.W., Mural,R.J. et al. The sequence of the human genome. *Science* **291**, 1304-1351 (2001).

33. ....Weiss,S.T., McLeod,H.L., Flockhart,D.A., Dolan,M.E., Benowitz,N.L. et al. Creating and evaluating genetic tests predictive of drug response. *Nat Rev Drug Discov.* **7**, 568-574 (2008).

34. ....Mallal,S., Phillips,E., Carosi,G., Molina,J.M., Workman,C. et al. HLA-B*5701 screening for hypersensitivity to abacavir. *N Engl J Med* **358**, 568-579 (2008).

35. ....Nebert,D.W. & Vesell,E.S. Advances in pharmacogenomics and individualized drug therapy: exciting challenges that lie ahead. *Eur. J Pharmacol* **500**, 267-280 (2004).

36. ....Ioannidis,J.P., Trikalinos,T.A., Ntzani,E.E., & Contopoulos-Ioannidis,D.G. Genetic associations in large versus small studies: an empirical assessment. *Lancet* **361**, 567-571 (2003).

37. ....Ntzani,E.E., Rizos,E.C., & Ioannidis,J.P. Genetic effects versus bias for candidate polymorphisms in myocardial infarction: case study and overview of large-scale evidence. *Am J Epidemiol* **165**, 973-984 (2007).

38. ....Lesnick,T.G., Papapetropoulos,S., Mash,D.C., Ffrench-Mullen,J., Shehadeh,L. et al. A genomic pathway approach to a complex disease: axon guidance and Parkinson disease. *PLoS. Genet* **3**, e98 (2007).

39. ....Donfack,J., Buchinsky,F.J., Post,J.C., & Ehrlich,G.D. Human susceptibility to viral infection: the search for HIV-protective alleles among Africans by means of genome-wide studies. AIDS Res Hum. *Retroviruses* **22**, 925-930 (2006).

40. ....McClurg,P., Pletcher,M.T., Wiltshire,T., & Su,A.I. Comparative analysis of haplotype association mapping algorithms. *BMC. Bioinformatics* **7**, 61 (2006).

41. ....Frazer,K.A., Eskin,E., Kang,H.M., Bogue,M.A., Hinds,D.A. et al. A sequence-based variation map of 8.27 million SNPs in inbred mouse strains. *Nature* **448**, 1050-1053 (2007).

42. ....Pletcher,M.T., McClurg,P., Batalov,S., Su,A.I., Barnes,S.W. et al. Use of a dense single nucleotide polymorphism map for in silico mapping in the mouse. *PLoS. Biol.* **2**, e393 (2004).

43. ....Guo,Y., Weller,P., Farrell,E., Cheung,P., Fitch,B. et al. In silico pharmacogenetics of warfarin metabolism. *Nat Biotechnol.* **24**, 531-536 (2006).

44. ....Guo,Y., Lu,P., Farrell,E., Zhang,X., Weller,P. et al. In silico and in vitro pharmacogenetic analysis in mice. *Proc. Natl. Acad Sci U. S. A* **104**, 17735-17740 (2007).

45. ....Nebert,D.W., Jorge-Nebert,L., & Vesell,E.S. Pharmacogenomics and "individualized drug therapy": high expectations and disappointing achievements. *Am J Pharmacogenomics.* **3**, 361-370 (2003).

46. ....Phipps,A.N., Stewart,J., Wright,B., & Wilson,I.D. Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between diet, gut microflora and substrate specificity. *Xenobiotica* **28**, 527-537 (1998).

47. ....Schrattenholz,A. & Ss,K., V What does systems biology mean for drug development? *Curr. Med Chem* **15**, 1520-1528 (2008).

48. ....Fostel,J.M. Future of toxicogenomics and safety signatures: balancing public access to data with proprietary drug discovery. *Pharmacogenomics.* **8**, 425-430 (2007).

49. ....Shenton,J.M., Chen,J., & Uetrecht,J.P. Animal models of idiosyncratic drug reactions. *Chem Biol. Interact.* **150**, 53-70 (2004).

50. ....Holt,M.P. & Ju,C. Mechanisms of drug-induced liver injury. *AAPS.* J **8**, E48-E54 (2006).

51. ....Larrey,D. Drug-induced liver diseases. *J Hepatol*. **32**, 77-88 (2000).

52. ....Watkins,P.B., Kaplowitz,N., Slattery,J.T., Colonese,C.R., Colucci,S.V. et al. Aminotransferase elevations in healthy adults receiving 4 grams of acetaminophen daily: a randomized controlled trial. *J. Am. Med. Assoc.* **296**, 87-93 (2006).

53. ....Lee,W.M. Acute liver failure in the United States. *Semin. Liver Dis.* **23**, 217-226 (2003).

54. ....Kaplowitz,N. Idiosyncratic drug hepatotoxicity. *Nat. Rev. Drug Discov.* **4**, 489-499 (2005).

55. ....Ju,C., Reilly,T.P., Bourdi,M., Radonovich,M.F., Brady,J.N. et al. Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol.* **15**, 1504-1513 (2002).

56. ....Liu,Z.X., Govindarajan,S., & Kaplowitz,N. Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. *Gastroenterology* **127**, 1760-1774 (2004).

57. ....Chia,R., Achilli,F., Festing,M.F., & Fisher,E.M. The origins and uses of mouse outbred stocks. *Nat. Genet.* **37**, 1181-1186 (2005).

58. ....James,L.P., Simpson,P.M., Farrar,H.C., Kearns,G.L., Wasserman,G.S. et al. Cytokines and toxicity in acetaminophen overdose. *J Clin. Pharmacol.* **45**, 1165-1171 (2005).

59. ....James,L.P., Kurten,R.C., Lamps,L.W., McCullough,S., & Hinson,J.A. Tumour necrosis factor receptor 1 and hepatocyte regeneration in acetaminophen toxicity: a kinetic study of proliferating cell nuclear antigen and cytokine expression. *Basic Clin. Pharmacol. Toxicol.* **97**, 8-14 (2005).

60. ....Liu,Z.X., Han,D., Gunawan,B., & Kaplowitz,N. Neutrophil depletion protects against murine acetaminophen hepatotoxicity. *Hepatology* **43**, 1220-1230 (2006).

61. ....Cover,C., Liu,J., Farhood,A., Malle,E., Waalkes,M.P. et al.

Pathophysiological role of the acute inflammatory response during acetaminophen hepatotoxicity. *Toxicol Appl. Pharmacol* **216**, 98-107 (2006).

62. ....Jaeschke,H. How relevant are neutrophils for acetaminophen hepatotoxicity? *Hepatology* **43**, 1191-1194 (2006).

63. ....Lawson,J.A., Farhood,A., Hopper,R.D., Bajt,M.L., & Jaeschke,H. The hepatic inflammatory response after acetaminophen overdose: role of neutrophils. *Toxicol Sci* **54**, 509-516 (2000).

64. ....Beck,J.A., Lloyd,S., Hafezparast,M., Lennon-Pierce,M., Eppig,J.T. et al. Genealogies of mouse inbred strains. *Nat. Genet.* **24**, 23-25 (2000).

65. ....Shenton,J.M., Chen,J., & Uetrecht,J.P. Animal models of idiosyncratic drug reactions. *Chem Biol. Interact.* **150**, 53-70 (2004).

66. ....Larrey,D. Drug-induced liver diseases. *J Hepatol.* **32**, 77-88 (2000).

67. ....Collins,F.S., Gray,G.M., & Bucher,J.R. Toxicology. Transforming environmental health protection. *Science* **319**, 906-907 (2008).

68. ....Ingelman-Sundberg,M. Pharmacogenomic biomarkers for prediction of severe adverse drug reactions. *N Engl J Med* **358**, 637-639 (2008).

69. ....Lanfear,D.E. & McLeod,H.L. Pharmacogenetics: using DNA to optimize drug therapy. *Am Fam. Physician* **76**, 1179-1182 (2007).

70. ....Tomalik-Scharte,D., Lazar,A., Fuhr,U., & Kirchheiner,J. The clinical role of genetic polymorphisms in drug-metabolizing enzymes. *Pharmacogenomics.* J **8**, 4-15 (2008).

71. ....Paigen,K. One hundred years of mouse genetics: an intellectual history. II. The molecular revolution (1981-2002). *Genetics* **163**, 1227-1235 (2003).

72. ....Wade,C.M. & Daly,M.J. Genetic variation in laboratory mice. *Nat. Genet.* **37**, 1175-1180 (2005).

73. ....Paigen,K. & Eppig,J.T. A mouse phenome project. *Mamm. Genome* **11**, 715-717 (2000).

74. ....Roberts,A., Pardo-Manuel,d., V, Wang,W., McMillan,L., & Threadgill,D.W. The polymorphism architecture of mouse genetic resources elucidated using genome-wide resequencing data: implications for QTL discovery and systems genetics. *Mamm. Genome* **18**, 473-481 (2007).

75. ....Lee,W.M. Acute liver failure in the United States. *Semin. Liver Dis*. **23**, 217-226 (2003).

76. ....Lee,W.M. Acetaminophen toxicity: changing perceptions on a social/medical issue. *Hepatology* **46**, 966-970 (2007).

77. ....Jaeschke,H. & Bajt,M.L. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol Sci* **89**, 31-41 (2006).

78. ....James,L.P., Simpson,P.M., Farrar,H.C., Kearns,G.L., Wasserman,G.S. et al. Cytokines and toxicity in acetaminophen overdose. *J Clin. Pharmacol* **45**, 1165-1171 (2005).

79. ....Kuffner,E.K., Temple,A.R., Cooper,K.M., Baggish,J.S., & Parenti,D.L. Retrospective analysis of transient elevations in alanine aminotransferase during long-term treatment with acetaminophen in osteoarthritis clinical trials. *Curr. Med Res Opin.* **22**, 2137-2148 (2006).

80. ....Bogue,M.A. & Grubb,S.C. The Mouse Phenome Project. *Genetica* **122**, 71-74 (2004).

81. ....Boorman,G.A., Blackshear,P.E., Parker,J.S., Lobenhofer,E.K., Malarkey,D.E. et al. Hepatic gene expression changes throughout the day in the Fischer rat: implications for toxicogenomic experiments. *Toxicol. Sci* **86**, 185-193 (2005).

82. ....Bergmeyer,H.U., Horder,M., & Rej,R. International Federation of Clinical Chemistry (IFCC) Scientific Committee, Analytical Section: approved recommendation (1985) on IFCC methods for the measurement of catalytic concentration of enzymes. Part 3. IFCC method for alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2*). J Clin. Chem Clin. Biochem* **24**, 481-495 (1986).

83. ....Mouton,P.R. Principles and Practices of Unbiased Stereology: An Introduction for Bioscientists(The Johns Hopkins University Press,2002).

84. ....Kulkarni,S.G., Pegram,A.A., & Smith,P.C. Disposition of acetaminophen and indocyanine green in cystic fibrosis-knockout mice. *AAPS. PharmSci*. **2**, E18 (2000).

85. ....Schadt,E.E., Monks,S.A., Drake,T.A., Lusis,A.J., Che,N. et al. Genetics of gene expression surveyed in maize, mouse and man. *Nature* **422**, 297-302 (2003).

86. ....Mitchell,J.R., Jollow,D.J., Potter,W.Z., Gillette,J.R., & Brodie,B.B. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther* **187**, 211-217 (1973).

87. ....Gonzalez,F.J. Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. *Mutat. Res* **569**, 101-110 (2005).

88. ....Nelson,M.R., Bacanu,S.A., Mosteller,M., Li,L., Bowman,C.E. et al. Genome-wide approaches to identify pharmacogenetic contributions to adverse drug reactions. *Pharmacogenomics. J*. In press (2008).

89. ....Kindmark,A., Jawaid,A., Harbron,C.G., Barratt,B.J., Bengtsson,O.F. et al. Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis. *Pharmacogenomics. J*(2007).

90. ....James,L.P., Lamps,L.W., McCullough,S., & Hinson,J.A. Interleukin 6 and hepatocyte regeneration in acetaminophen toxicity in the mouse. *Biochem Biophys. Res Commun*. **309**, 857-863 (2003).

91. ....Bourdi,M., Eiras,D.P., Holt,M.P., Webster,M.R., Reilly,T.P. et al. Role of IL-6 in an IL-10 and IL-4 double knockout mouse model uniquely susceptible to acetaminophen-induced liver injury. *Chem Res Toxicol* **20**, 208-216 (2007).

92. ....Ishida,Y., Kondo,T., Ohshima,T., Fujiwara,H., Iwakura,Y. et al. A pivotal involvement of IFN-gamma in the pathogenesis of acetaminophen-induced acute liver injury. *FASEB J* **16**, 1227-1236 (2002).

93. ....Gardner,C.R., Laskin,J.D., Dambach,D.M., Sacco,M., Durham,S.K. et al. Reduced hepatotoxicity of acetaminophen in mice lacking inducible nitric oxide synthase: potential role of tumor necrosis factor-alpha and interleukin-10. *Toxicol. Appl. Pharmacol.* **184**, 27-36 (2002).

94. ....Ju,C., Reilly,T.P., Bourdi,M., Radonovich,M.F., Brady,J.N. et al. Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol.* **15**, 1504-1513 (2002).

95. ....Knight,T.R. & Jaeschke,H. Peroxynitrite formation and sinusoidal endothelial cell injury during acetaminophen-induced hepatotoxicity in mice. *Comp Hepatol.* **3** Suppl 1, S46 (2004).

96. ....Limaye,P.B., Apte,U.M., Shankar,K., Bucci,T.J., Warbritton,A. et al. Calpain released from dying hepatocytes mediates progression of acute liver injury induced by model hepatotoxicants. *Toxicol. Appl. Pharmacol*. **191**, 211-226 (2003).

97. ....Limaye,P.B., Bhave,V.S., Palkar,P.S., Apte,U.M., Sawant,S.P. et al. Upregulation of calpastatin in regenerating and developing rat liver: role in resistance against hepatotoxicity. *Hepatology* **44**, 379-388 (2006).

98. ....Kimura,K., Nagaki,M., Kakimi,K., Saio,M., Saeki,T. et al. Critical role of CD44 in hepatotoxin-mediated liver injury. *J Hepatol*.In press (2008).

99. ....Rouschop,K.M., Claessen,N., Pals,S.T., Weening,J.J., & Florquin,S. CD44 disruption prevents degeneration of the capillary network in obstructive nephropathy via reduction of TGF-beta1-induced apoptosis. *J Am Soc Nephrol* **17**, 746-753 (2006).

100. ...International Program on Chemical Safety Biomarkers and risk assessment concepts and principles (World Health Organization, Geneva, Switzerland, 1993).

101. ...Casciano,D.A. & Woodcock,J. Empowering microarrays in the regulatory setting. *Nat. Biotechnol*. **24**, 1103 (2006).

102. ...Dix,D.J., Gallagher,K., Benson,W.H., Groskinsky,B.L., McClintock,J.T. et

al. A framework for the use of genomics data at the EPA. *Nat Biotechnol.* **24**, 1108-1111 (2006).

103. ...Fry,R.C., Navasumrit,P., Valiathan,C., Svensson,J.P., Hogan,B.J. et al. Activation of inflammation/NF-kappaB signaling in infants born to arsenic-exposed mothers. *PLoS. Genet* **3**, e207 (2007).

104. ...Castle,A.L., Carver,M.P., & Mendrick,D.L. Toxicogenomics: a new revolution in drug safety. *Drug Discov. Today* **7**, 728-736 (2002).

105. ...Ganter,B., Zidek,N., Hewitt,P.R., Muller,D., & Vladimirova,A. Pathway analysis tools and toxicogenomics reference databases for risk assessment. *Pharmacogenomics.* **9**, 35-54 (2008).

106. ...Rininger,J.A., DiPippo,V.A., & Gould-Rothberg,B.E. Differential gene expression technologies for identifying surrogate markers of drug efficacy and toxicity. *Drug Discov. Today* **5**, 560-568 (2000).

107. ...Waters,M., Stasiewicz,S., Merrick,B.A., Tomer,K., Bushel,P. et al. CEBS--Chemical Effects in Biological Systems: a public data repository integrating study design and toxicity data with microarray and proteomics data. *Nucleic Acids Res* **36**, D892-D900 (2008).

108. ...Mattingly,C.J., Rosenstein,M.C., Davis,A.P., Colby,G.T., Forrest,J.N., Jr. et al. The comparative toxicogenomics database: a cross-species resource for building chemical-gene interaction networks. *Toxicol Sci* **92**, 587-595 (2006).

109. ...Feero,W.G., Guttmacher,A.E., & Collins,F.S. The genome gets personal—almost. *J. Am. Med. Assoc.* **299**, 1351-1352 (2008).

110. ...Chesler,E.J., Lu,L., Shou,S., Qu,Y., Gu,J. et al. Complex trait analysis of gene expression uncovers polygenic and pleiotropic networks that modulate nervous system function. *Nat. Genet.* **37**, 233-242 (2005).

111. ...Gatti,D., Maki,A., Chesler,E.J., Kirova,R., Kosyk,O. et al. Genome-level analysis of genetic regulation of liver gene expression networks. *Hepatology* **46**, 548-557 (2007).

112. ...Svenson,K.L., Von Smith,R., Magnani,P.A., Suetin,H.R., Paigen,B. et al.

Multiple Trait Measurements in 43 Inbred Mouse Strains Captures the Phenotypic Diversity Characteristic of Human Populations. *J Appl Physiol*(2007).

113. ...Benjamini,Y. & Liu,W. A step-down multiple hypotheses testing procedure that controls the false discovery rate under independence. *Journal of Statistical Planning and Inference* **82**, 163-170 (1999).

114. ...Draghici,S., Khatri,P., Tarca,A.L., Amin,K., Done,A. et al. A systems biology approach for pathway level analysis. *Genome Res* **17**, 1537-1545 (2007).

115. ...Hinson,J.A., Reid,A.B., McCullough,S.S., & James,L.P. Acetaminophen-induced hepatotoxicity: role of metabolic activation, reactive oxygen/nitrogen species, and mitochondrial permeability transition. *Drug Metab Rev*. **36**, 805-822 (2004).

116. ...James,L.P., Mayeux,P.R., & Hinson,J.A. Acetaminophen-induced hepatotoxicity. *Drug Metab Dispos* **31**, 1499-1506 (2003).

117. ...Foley,J.F., Collins,J.B., Umbach,D.M., Grissom,S., Boorman,G.A. et al. Optimal sampling of rat liver tissue for toxicogenomic studies. *Toxicol Pathol* **34**, 795-801 (2006).

118. ...Beyer,R.P., Fry,R.C., Lasarev,M.R., McConnachie,L.A., Meira,L.B. et al. Multicenter study of acetaminophen hepatotoxicity reveals the importance of biological endpoints in genomic analyses. *Toxicol Sci* **99**, 326-337 (2007).

119. ...Kwon,Y.H., Jovanovic,A., Serfas,M.S., & Tyner,A.L. The Cdk inhibitor p21 is required for necrosis, but it inhibits apoptosis following toxin-induced liver injury. *J Biol Chem* **278**, 30348-30355 (2003).

120. ...Streetz,K.L., Wustefeld,T., Klein,C., Kallen,K.J., Tronche,F. et al. Lack of gp130 expression in hepatocytes promotes liver injury. *Gastroenterology* **125**, 532-543 (2003).

121. ...Wuestefeld,T., Klein,C., Streetz,K.L., Betz,U., Lauber,J. et al. Interleukin-6/glycoprotein 130-dependent pathways are protective during liver regeneration. *J Biol Chem* **278**, 11281-11288 (2003).

122. ...Richards,C.D., Shoyab,M., Brown,T.J., & Gauldie,J. Selective regulation of metalloproteinase inhibitor (TIMP-1) by oncostatin M in fibroblasts in culture. *J Immunol.* **150**, 5596-5603 (1993).

123. ...Li,W.Q. & Zafarullah,M. Oncostatin M up-regulates tissue inhibitor of metalloproteinases-3 gene expression in articular chondrocytes via de novo transcription, protein synthesis, and tyrosine kinase- and mitogen-activated protein kinase-dependent mechanisms. *J Immunol.* **161**, 5000-5007 (1998).

124. ...Masubuchi,Y., Bourdi,M., Reilly,T.P., Graf,M.L., George,J.W. et al. Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease. *Biochem Biophys. Res Commun.* **304**, 207-212 (2003).

125. ...Nakamura,K., Nonaka,H., Saito,H., Tanaka,M., & Miyajima,A. Hepatocyte proliferation and tissue remodeling is impaired after liver injury in oncostatin M receptor knockout mice. *Hepatology* **39**, 635-644 (2004).

126. ...Kendig,E.L., Schneider,S.N., Clegg,D.J., Genter,M.B., & Shertzer,H.G. Over-the-counter analgesics normalize blood glucose and body composition in mice fed a high fat diet. *Biochem Pharmacol* **76**, 216-224 (2008).

127. ...Shertzer,H.G., Schneider,S.N., Kendig,E.L., Clegg,D.J., D'Alessio,D.A. et al. Acetaminophen normalizes glucose homeostasis in mouse models for diabetes. *Biochem Pharmacol* **75**, 1402-1410 (2008).

128. ...Coen,M., Ruepp,S.U., Lindon,J.C., Nicholson,J.K., Pognan,F. et al. Integrated application of transcriptomics and metabonomics yields new insight into the toxicity due to paracetamol in the mouse. *J Pharm Biomed Anal* **35**, 93-105 (2004).

129. ...Festing,M.F. Experimental approaches to the determination of genetic variability. *Toxicol. Lett.* **120**, 293-300 (2001).

130. ...Ideraabdullah,F.Y., Casa-Esperon,E., Bell,T.A., Detwiler,D.A., Magnuson,T. et al. Genetic and haplotype diversity among wild-derived mouse inbred strains. *Genome Res* **14**, 1880-1887 (2004).

131. ...Klabunde,T. & Hessler,G. Drug design strategies for targeting G-protein-

coupled receptors. *Chembiochem*. **3**, 928-944 (2002).

132. ...Koch-Weser,J. The serum level approach to individualization of drug dosage. *Eur. J Clin Pharmacol* **9**, 1-8 (1975).

133. ...Welch,K.D., Reilly,T.P., Bourdi,M., Hays,T., Pise-Masison,C.A. et al. Genomic identification of potential risk factors during acetaminophen-induced liver disease in susceptible and resistant strains of mice. *Chem Res Toxicol* **19**, 223-233 (2006).

134. ...Welch,K.D., Wen,B., Goodlett,D.R., Yi,E.C., Lee,H. et al. Proteomic identification of potential susceptibility factors in drug-induced liver disease. *Chem Res Toxicol* **18**, 924-933 (2005).

135. ...Prescott,L.F. Kinetics and metabolism of paracetamol and phenacetin. *Br. J Clin Pharmacol* **10** Suppl 2, 291S-298S (1980).

136. ...Mitchell,J.R., Thorgeirsson,S.S., Potter,W.Z., Jollow,D.J., & Keiser,H. Acetaminophen-induced hepatic injury: protective role of glutathione in man and rationale for therapy. *Clin Pharmacol Ther* **16**, 676-684 (1974).

137. ...Bagnall,W.E., Kelleher,J., Walker,B.E., & Losowsky,M.S. The gastrointestinal absorption of paracetamol in the rat. *J Pharm Pharmacol* **31**, 157-160 (1979).

138. ...Josting,D., Winne,D., & Bock,K.W. Glucuronidation of paracetamol, morphine and 1-naphthol in the rat intestinal loop. *Biochem Pharmacol* **25**, 613-616 (1976).

139. ...Wang,L.H., Rudolph,A.M., & Benet,L.Z. Pharmacokinetic studies of the disposition of acetaminophen in the sheep maternal-placental-fetal unit. *J Pharmacol Exp Ther* **238**, 198-205 (1986).

140. ...Price,V.F. & Jollow,D.J. Strain differences in susceptibility of normal and diabetic rats to acetaminophen hepatotoxicity. *Biochem Pharmacol* **35**, 687-695 (1986).

141. ...Price,V.F. & Jollow,D.J. Increased resistance of diabetic rats to

acetaminophen-induced hepatotoxicity. *J Pharmacol Exp Ther* **220**, 504-513 (1982).

142. ...Kim,H.J., Rozman,P., & Klaassen,C.D. Acetaminophen does not decrease hepatic 3'-phosphoadenosine 5'-phosphosulfate in mice. *J Pharmacol Exp Ther* **275**, 1506-1511 (1995).

143. ...Liu,L. & Klaassen,C.D. Different mechanism of saturation of acetaminophen sulfate conjugation in mice and rats. *Toxicol Appl Pharmacol* **139**, 128-134 (1996).

144. ...Vaquero,J., Belanger,M., James,L., Herrero,R., Desjardins,P. et al. Mild hypothermia attenuates liver injury and improves survival in mice with acetaminophen toxicity. *Gastroenterology* **132**, 372-383 (2007).

145. ...Reid,A.B., Kurten,R.C., McCullough,S.S., Brock,R.W., & Hinson,J.A. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. *J Pharmacol Exp Ther* **312**, 509-516 (2005).

146. ...Wilson,S.G., Bryant,C.D., Lariviere,W.R., Olsen,M.S., Giles,B.E. et al. The heritability of antinociception II: pharmacogenetic mediation of three over-the-counter analgesics in mice. *J Pharmacol Exp Ther* **305**, 755-764 (2003).

147. ...Bonnefont,J., Daulhac,L., Etienne,M., Chapuy,E., Mallet,C. et al. Acetaminophen recruits spinal p42/p44 MAPKs and GH/IGF-1 receptors to produce analgesia via the serotonergic system. *Mol Pharmacol* **71**, 407-415 (2007).

148. ...Aleksunes,L.M., Slitt,A.M., Cherrington,N.J., Thibodeau,M.S., Klaassen,C.D. et al. Differential expression of mouse hepatic transporter genes in response to acetaminophen and carbon tetrachloride. *Toxicol Sci* **83**, 44-52 (2005).

149. ...McPhail,M.E., Knowles,R.G., Salter,M., Dawson,J., Burchell,B. et al. Uptake of acetaminophen (paracetamol) by isolated rat liver cells. *Biochem Pharmacol* **45**, 1599-1604 (1993).

150. Konig,J., Rost,D., Cui,Y., & Keppler,D. Characterization of the human

multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology* **29**, 1156-1163 (1999).

151. ...Gunawan,B.K., Liu,Z.X., Han,D., Hanawa,N., Gaarde,W.A. et al. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology* **131**, 165-178 (2006).

152. ...Liu,Z.X. & Kaplowitz,N. Role of innate immunity in acetaminophen-induced hepatotoxicity. *Expert. Opin. Drug Metab Toxicol* **2**, 493-503 (2006).

153. ...Nelson,S.D. & Bruschi,S.A. Mechanisms of acetaminophen-induced liver disease in Drug-Induced Liver Disease (eds. Kaplowitz,N. & DeLeve,L.D.) (New York and Basel, Marcel Dekker, 2002).

154. ...Powell,C.L., Kosyk,O., Bradford,B.U., Parker,J.S., Lobenhofer,E.K. et al. Temporal correlation of pathology and DNA damage with gene expression in a choline-deficient model of rat liver injury. *Hepatology* **42**, 1137-1147 (2005).

155. ...Stylianou,I.M., Tsaih,S.W., Dipetrillo,K., Ishimori,N., Li,R. et al. Complex Genetic Architecture Revealed by Analysis of HDL in Chromosome Substitution Strains and F2 Crosses. *Genetics*(2006).

156. ...Cho,H.Y., Jedlicka,A.E., Reddy,S.P., Zhang,L.Y., Kensler,T.W. et al. Linkage analysis of susceptibility to hyperoxia. Nrf2 is a candidate gene. *Am J Respir. Cell Mol. Biol.* **26**, 42-51 (2002).

157. ...McClurg,P., Janes,J., Wu,C., Delano,D.L., Walker,J.R. et al. Genomewide association analysis in diverse inbred mice: power and population structure. *Genetics* **176**, 675-683 (2007).

158. ...Churchill,G.A., Airey,D.C., Allayee,H., Angel,J.M., Attie,A.D. et al. The Collaborative Cross, a community resource for the genetic analysis of complex traits. *Nat. Genet.* **36**, 1133-1137 (2004).

159. ...Iraqi,F.A., Churchill,G., & Mott,R. The Collaborative Cross, developing a resource for mammalian systems genetics: A status report of the Wellcome Trust cohort. *Mamm. Genome*(2008).

160. ...Ito,Y., Abril,E.R., Bethea,N.W., McCuskey,M.K., & McCuskey,R.S. Dietary

steatotic liver attenuates acetaminophen hepatotoxicity in mice. *Microcirculation*. **13**, 19-27 (2006).

161. ...Shayiq,R.M., Roberts,D.W., Rothstein,K., Snawder,J.E., Benson,W. et al. Repeat exposure to incremental doses of acetaminophen provides protection against acetaminophen-induced lethality in mice: an explanation for high acetaminophen dosage in humans without hepatic injury. *Hepatology* **29**, 451-463 (1999).

162. ...Yang,L., Allen,B.C., & Thomas,R.S. BMDExpress: a software tool for the benchmark dose analyses of genomic data. *BMC. Genomics* **8**, 387 (2007).

163. ...Albano,E., Rundgren,M., Harvison,P.J., Nelson,S.D., & Moldeus,P. Mechanisms of N-acetyl-p-benzoquinone imine cytotoxicity. *Mol Pharmacol* **28**, 306-311 (1985).

164. ...Ma,X.Z., Jin,T., Sakac,D., Fahim,S., Zhang,X. et al. Abnormal splicing of SHP-1 protein tyrosine phosphatase in human T cells. Implications for lymphomagenesis. *Exp Hematol*. **31**, 131-142 (2003).

165. ...Godar,S., Ince,T.A., Bell,G.W., Feldser,D., Donaher,J.L. et al. Growth-inhibitory and tumor- suppressive functions of p53 depend on its repression of CD44 expression. *Cell* **134**, 62-73 (2008).

166. ...Wang,K., Li,M., & Bucan,M. Pathway-Based Approaches for Analysis of Genomewide Association Studies. *Am J Hum. Genet* **81**, (2007).

167. ...Subramanian,A., Tamayo,P., Mootha,V.K., Mukherjee,S., Ebert,B.L. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad Sci U. S. A* **102**, 15545-15550 (2005).

168. ...Deng,X., Stachlewitz,R.F., Liguori,M.J., Blomme,E.A., Waring,J.F. et al. Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. *J Pharmacol Exp Ther* **319**, 1191-1199 (2006).

169. ...Buchweitz,J.P., Ganey,P.E., Bursian,S.J., & Roth,R.A. Underlying

endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy? *J Pharmacol Exp Ther* **300**, 460-467 (2002).

170. ...Shaw,P.J., Hopfensperger,M.J., Ganey,P.E., & Roth,R.A. Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. *Toxicol Sci* **100**, 259-266 (2007).