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Title Page

ANALYSIS OF GLOBAL AND ADME GENE EXPRESSION IN THE PROGRESSIVE STAGES OF HUMAN NON-ALCOHOLIC FATTY LIVER DISEASE

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Running Title Page

Running Title: ADME gene expression in non-alcoholic fatty liver disease.

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Abbreviations:

NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ADME, absorption, distribution, metabolism and excretion; ABC transporter, adenosine triphosphate-binding-cassette transporter; SLC transporter, solute carrier transporter; DME, drug metabolizing enzyme; P450, cytochrome P-450; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LIMMA, linear model for microarrays; PCA, principal component analysis.

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Abstract.

Non-alcoholic fatty liver disease (NAFLD) is characterized by a series of pathological changes that range from simple fatty liver to non-alcoholic steatohepatitis (NASH). The objective of this study is to describe changes in global gene expression associated with the progression of human NAFLD. This study is focused on the expression levels of genes responsible for the absorption, distribution, metabolism and elimination (ADME) of drugs. Differential gene expression between three clinically defined pathological groups; normal, steatosis and NASH was analyzed. Genome-wide mRNA levels in samples of human liver tissue were assayed with Affymetrix GeneChip Human 1.0ST arrays. A total of 11,633 genes exhibited altered expression out of 33,252 genes at a 5 % false discovery rate. The majority of gene expression changes occurred in the progression from steatosis to NASH. Principal component analysis (PCA) revealed that hepatic disease status was the major determinant of differential ADME gene expression rather than age or sex of sample donors. Among the 515 drug transporters and 258 drug metabolizing enzymes (DMEs) examined, uptake transporters but not efflux transporters or DMEs were significantly over-represented in the number of genes downregulated. These results suggest that uptake transporter genes are coordinately targeted for downregulation at the global level during the pathological development of NASH and that these patients may have decreased drug uptake capacity. This coordinated regulation of uptake transporter genes is indicative of a hepatoprotective mechanism acting to prevent accumulation of toxic intermediates in disease-compromised hepatocytes.

Introduction.

Non-alcoholic fatty liver disease (NAFLD) is a progressive disease of worldwide significance that currently afflicts 30–40 % of the United States population (Ali and Cusi, 2009). Almost half of all individuals with NAFLD may actually have the more severe stage of NASH (Ali and Cusi, 2009). Globally, the prevalence of NAFLD is expected to approach that of the United States due to the spread of western lifestyle (Bellentani, et al., 2010). The mechanisms responsible for the transition from simple fatty liver to NASH have yet to be elucidated. However, recent endeavors have characterized the progression of human NAFLD through a two hit model of pathogenesis (Day and James, 1998). According to this model, NAFLD originates as steatosis through a 'first hit' characterized by the abnormal accumulation of lipids within hepatocytes (Day, 2002;Rombouts and Marra, 2010). The 'second hit' required for advancement to NASH, is thought to be initiated by excess production of reactive oxygen species (ROS) and pro-inflammatory cytokines (Pessayre, et al., 2004). Gene expression studies of human NAFLD have been limited to themes of the first and second hit. There is a focus on obesity-related gene expression changes in NAFLD in addition to inflammatory and immune system components of the liver (Baranova, et al., 2005;Bertola, et al., 2010;Younossi, et al., 2005b). However, there is a lack of studies on expression changes of genes related to the absorption, distribution, metabolism and elimination (ADME) processes in progressive human NAFLD.

Gene expression changes associated with ADME processes may alter drug transport and distribution in NAFLD patients resulting in an elevated risk of ADRs and altered

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drug bioavailability. The topic of ADME gene expression changes in the NAFLD patient population is of interest as this clinical group is often subjected to multiple prescription medications due to co-morbidities related to the metabolic syndrome such as type 2 diabetes mellitus, hypertension and dyslipidemia (Portincasa, et al., 2006). Combination drug therapies put this patient population at significant risk of adverse drug reactions (ADRs). ADRs are a frequent occurrence in today's medical field and represent a common cause of death in hospitalized patients (Lazarou, et al., 1998).

The components of ADME genes include phase I and II drug metabolizing enzymes (DMEs) as well as, the so called phase 0 uptake and phase III efflux transporters (Szakacs, et al., 2008). Phase I and II DMEs modulate the pharmacokinetics of endogenous and exogenous compounds in the body. The expression of hepatic phase I DMEs, such as the cytochrome P-450 family members, has been shown to be significantly altered in human NAFLD by previous studies (Fisher, et al., 2009c). Transport proteins, considered the phase 0 and III components of ADME events, are recognized to have critical roles in the vectorial transport of drugs across cell membranes. Two main categories of transporters in the liver include uptake, generally known as the solute carrier transporters (SLC) and efflux, generally known as the ATP-binding cassette (ABC) transporters. In the liver, efflux transporters residing on the sinusoidal and canalicular membranes of hepatocytes are responsible for substrate excretion into plasma and bile, respectively, whereas uptake transporters are primarily responsible for drug uptake into the hepatocyte from blood. Gene expression changes of SLC uptake transporter family members have previously been demonstrated in our

laboratory using a rodent model of NAFLD (Fisher, et al., 2009a). Other members of our laboratory have examined efflux transporter expression changes in a murine model of cholestasis. A hepatoprotective response to cholestasis was observed at the transcriptional level through the upregulation of certain sinusoidal efflux transporters and downregulation of specific uptake transporters (Lickteig, et al., 2007b).

Patterns of gene expression changes provide evidence of transcriptional mechanisms of regulation. Coordinate regulation has been previously reported by investigators studying nuclear receptors (Eloranta and Meier, 2005;Maglich, et al., 2002;Pascussi, et al., 2008). Upon activation, nuclear receptors induce a small gene battery consisting of both phase I and II metabolizing enzymes and phase 0 and III transporters (Kohle and Bock, 2007). Coordinate regulation at the global expression level for ADME genes has not been shown but could have profound effects upon hepatic function and toxicity (Kohle and Bock, 2009). Principal component analysis (PCA) is a method utilized to examine patterns of expression changes in genes. PCA simplifies complex gene array data by analyzing the variance. Patterns in ADME genes are analyzed in this study using PCA. The current study was designed to provide a comprehensive analysis of gene expression changes among DMEs and transporters across the progression of human NAFLD.

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Materials and Methods.

Human Liver Samples. Human liver tissue was previously acquired from the NIH funded Liver Tissue Cell Distribution System at the University of Minnesota, Virginia Commonwealth University and the University of Pittsburgh. Clinical and demographic information of these human liver samples has been described previously (Fisher, et al., 2009c). The samples were diagnosed as normal (n = 19), steatotic (n = 10), NASH with fatty liver (n = 9) and NASH without fatty liver (n = 7). NAFLD activity scoring (NAS) categorization was done by a Liver Tissue Cell Distribution System medical pathologist (Kleiner, et al., 2003). Steatosis was diagnosed by >10% fat deposition within hepatocytes without inflammation or fibrosis. NASH with fatty liver was characterized by >5% fat deposition with accompanied inflammation and fibrosis. NASH without fatty liver was distinguished by <5% fat deposition and increased inflammation and fibrosis.

Total RNA Isolation. Total RNA was isolated from the human liver samples using RNAzol B reagent (Tel-test Inc., Friendswood, TX). Total RNA was purified using the RNeasy Mini Qiagen purification kit (Valencia, CA) according to manufacturer's recommendations. RNA integrity was assessed using ethidium bromide staining following agarose gel electrophoresis. Concentration of the total RNA was determined using a Thermo Scientific Nanodrop 1000 spectrophotometer (Waltham, MA). The quality of the 18S and the 28S ribosomal bands were further characterized on an Agilent Bioanalyzer 2100 (Santa Clara, CA) using the Eukaryotic Total RNA Nano Assay with a 1 µL volume of purified total RNA from each sample. The RNA integrity numbering (RIN) system was used to assess the quality of the total RNA samples with a

RIN number of 1 being the most degraded and unusable form of total RNA while a RIN number of 10 represented the most complete and non-degraded form of the RNA.

Microarray Gene Expression Analysis. RNA was processed per manufacturer's protocol (Affymetrix GeneChip Whole Transcript (WT) Sense Target Labeling) for hybridization onto microarrays (Affymetrix GeneChip Human 1.0 ST Arrays). Array hybridization, washing and scanning was performed according to manufacturer's recommendations. All microarray data, archiving, and analysis were generated by the Genomics Core Facility at the Arizona Cancer Center. The array data was deposited into the ArrayExpress public repository for microarray data and is accessible under the accession number E-MEXP-3291 (ArrayExpress Archive, 2011). The differential expression of 33,252 global genes among three diagnosis groups (normal, steatosis, and NASH) was tested using the linear models for microarray data (LIMMA) software package in Bioconductor (Smyth GK, 2011). Pairwise comparisons between diagnosis groups were performed using LIMMA. Benjamini and Hochberg's method was used to control the false discovery rate at the level of 0.05 in order to correct for multiple hypothesis testing (Benjamini and Hochberg, 1995). For the purpose of the statistical tests employed in this study, NASH fatty and NASH not fatty samples were combined due to the lack of mechanistic differences between these two states despite histological differentiation. A pairwise comparison analysis of the gene expression at the 0.01 level of significance was performed to compare steatosis versus NASH fatty and NASH fatty versus NASH not fatty. This was done in order to demonstrate the lack of mechanistic

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differentiation between the two NASH categories and justify the combination of all NASH specimens into one group (Supplemental Figure 3).

ADME Gene Analysis. A list of 258 drug metabolizing enzymes (DMEs) and 515 transporter genes was compiled from literature sources. Separate lists were compiled for the subsequent analysis of 437 uptake transporter genes, 60 efflux transporter genes and 18 transporters classified as others. Differentially expressed genes are represented in Venn diagrams for each of the three pairwise comparisons for the global, DME and transporter categories (Figure 2). Distribution graphs were generated to show the proportion of genes tested among differentially expressed ADME gene categories. Categories were tested and if found to have a proportion of genes that showed greater representation in up or downregulation compared to the proportion of a randomly tested set of genes the same size are considered over-represented. The probability of acquiring a higher proportion of differentially expressed genes in a gene subset of DMEs or transporters was calculated from the simulated distribution (Figure 3). Heat maps were generated to visualize hierarchical clustering between patient diagnosis groups and gene expression levels (Figure 4). Principal component analysis (PCA) was used to simplify the complex data sets of gene categories by analyzing the components with the greatest amount of variance. Graphical representations of the first and second components of the entire global gene set and each ADME gene category show the similarities and differences in gene expression for the different groups of diagnosis (Figure 1 & Figure 5).

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Quantitative Reverse Transcription-Polymerase Chain Reaction (TaqMan) Validation Analysis. Validation of CYP1A2, CYP2D6, CYP2E1, and CYP2A6 mRNA levels was conducted using cDNA from the same set of human liver samples plus additional samples of each disease state that were categorized in a previous study (Fisher, et al., 2009c). Human liver tissue samples for these experiments were diagnosed as normal (n = 20), steatotic (n = 12), and NASH (n = 22). The cDNA was analyzed using gene-specific TaqMan primer/probe sets (Applied Biosystems, Foster City, CA). The ABI 7900HT real-time polymerase chain reaction system (Applied Biosciences) was utilized for these experiments using manufacturer's protocol for the assays. Reactions with the specific primer/probes for the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used as an endogenous control. The relation between the $\log_2$ microarray signal and raw –Ct values were plotted and examined for linearity for each selected gene (Supplemental Figures 1 and 2).

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Results.

RNA Integrity Numbers (RINs) for Human Liver Total RNA. The Eukaryotic Total RNA Nano Assay using the Agilent Bioanalyzer 2100 revealed RIN values for normal liver samples to be in the range of 7.1-10. Values for steatosis samples ranged from 8.2-10. RIN values of 5.2-9.9 were observed for NASH Fatty and 5.5-8.1 for NASH Not Fatty samples. These RIN values were found to be adequate for application of these samples to the Affymetrix GeneChip Whole Transcript (WT) Sense Target Labeling protocol.

Principal Component Analysis (PCA) of Global Gene Expression. To reveal the major factors correlated with gene expression changes, PCA was utilized. The first two principal components demonstrated that 34% of all expression variance is correlated with disease diagnosis but not with sex or age (Figure 1). In terms of the principal components, 22% of the total variance is represented by the first principal component and 12% by the second principal component (Figure 1). For the purpose of this study future analyses will only take into account the factor of diagnosis as this has been demonstrated by PCA to be the predominant contributor to the variance in the gene data set (Figure 1).

Differential Gene Expression of Global and ADME Genes. Gene expression analysis of the total 33,252 annotated and un-annotated global genes revealed 11,633 differentially expressed genes at the 0.05 level of significance among three pairwise comparisons (normal versus steatosis, steatosis versus NASH, and normal versus NASH) (Table 1, Figure 2). A total of 1,277 gene expression alterations (3.8% of all global genes) were

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revealed in the normal versus steatosis comparison (Table 1, Figure 2). 5,746 expression changes (17.3%) were discovered in the steatosis versus NASH comparison while 9,724 genes were differentially expressed (29.2%) in the normal versus NASH comparison (Table 1, Figure 2). 21,619 genes or 65% of all global gene expression was not changed (Table 1, Figure 2). This analysis demonstrates a greater magnitude of global gene expression changes in the samples that have progressed to NASH (Table 1, Figure 2).

Analysis of 258 DME genes revealed only 3 differentially expressed genes (1.2% of DME genes) in the normal versus steatosis comparison (Table 1, Figure 2, Supplemental Table 2). While expression of 57 genes (22.1% of DME genes) was changed in the comparison between steatosis and NASH, 81 genes (31.3% of DME genes) were changed in expression between normal and NASH (Table 1, Figure 2, Supplemental Table 2). The expression of a total of 166 DME genes (64.3%) remained unchanged (Table 1, Figure 2). Tests for enrichment of the DME category for expression changes in either up or downregulation revealed no over-representation in the distribution histogram (Figure 3).

A sum of 25 transporter genes demonstrated expression alterations out of 515 (4.8% of transporter genes) in the normal versus steatosis comparison (Table 1, Figure 2). While 150 expression changes (29.1%) occurred in the steatosis versus NASH comparison, 210 expression changes in total (40.8%) were observed in the normal versus NASH comparison (Table 1, Figure 2). The expression of 130 transporters (25.2%) remained

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unchanged (Table 1, Figure 2). Interestingly, the transporter gene category as a whole was significantly over-represented in a test among genes downregulated in expression for each of the NASH pairwise comparisons according to the transporter distribution histogram (Figure 3).

In the analysis of the efflux transporter gene category, 17 efflux genes showed altered expression in the steatosis versus NASH comparison analysis while 19 expression changes were observed in the normal versus NASH comparison. Only one efflux transporter was differentially expressed in the normal versus steatosis comparison (Supplemental Table 3). No over-representation was found for the 60 efflux transporter genes in either up or downregulation (Figure 3). For the uptake transporter gene category, 112 genes exhibited altered expression in the steatosis versus NASH comparison while only 22 were altered in expression in the normal versus steatosis comparison (Supplemental Table 2). A total of 162 uptake transporter genes were altered in the normal versus NASH comparison of which many also revealed expression changes in the steatosis versus NASH comparison (Supplemental Table 2). The distribution analysis of 437 uptake transporter genes revealed a strong over-representation not seen in the analysis of the efflux transporter genes (Figure 3). The over-representation among downregulated uptake transporter genes was observed in both the steatosis versus NASH and normal versus NASH comparisons (Figure 3). The normal versus steatosis comparison did not demonstrate any over-representation of genes downregulated in expression for either the uptake or the efflux transporter categories (Figure 3).

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Hierarchical Clustering Analysis of ADME Gene Categories. Hierarchical clustering revealed that the expression profile of transporter genes is capable of accurately assigning the pathological diagnosis of liver samples. Clustering of downregulated genes is represented as green blocks in the hierarchical clustering diagrams while upregulated genes are represented as red blocks (Figure 4). The NASH samples in the hierarchical clustering model form two distinctive clusters within the heat map with only one outlying NASH sample. In contrast, the DME gene expression is not sufficient to separate out NASH samples from the normal and steatotic groups in the hierarchical clustering model. Neither gene set is sufficient to distinguish specimens diagnosed as normal from those with steatosis (Figure 4).

Principal Component Analysis (PCA) of ADME Gene Categories. PCA of the transporter gene category shows a clear partition of NASH specimens from those diagnosed as normal and steatotic (Figure 5). In contrast more overlap is observed between the sample clusters in the DME PCA (Figure 5). This overlap makes it difficult to distinguish some NASH specimens from normal and steatotic in the DME genes. PCA of both the uptake and efflux transporter gene categories demonstrates distinctive clustering and division of NASH samples from those diagnosed as normal and steatotic (Figure 5).

Microarray Validation. Microarray data was correlated against raw quantitative RT-PCR data generated from the same set of human samples for validation of the microarray data. CYP1A2, CYP2D6, CYP2E1, and CYP2A6 were chosen for validation based upon

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data from a previous study by our laboratory using the same set of human liver specimens (Fisher, et al., 2009c). Normalized microarray expression (log_2 signal) was compared to quantitative RT-PCR data. All of the P450 validated genes demonstrated a corresponding pattern of fold change for up or downregulation similar to that seen in the microarray. The microarray log_2 signals and quantitative RT-PCR –Ct values from both sets of data were analyzed and found to be in good correlation for the four CYPs differentially expressed in NASH (Supplemental Figures 1 and 2).

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Discussion.

Human NAFLD has gained increasing attention as one of the more prominent chronic liver diseases of the decade, and it has raised clinical concerns due to its association with the pandemic of obesity and type 2 diabetes mellitus (Ali and Cusi, 2009;de Alwis and Day, 2008). The more severe stage of NAFLD has been associated with end-stage liver diseases such as cryptogenic cirrhosis and hepatocellular carcinoma. An estimated 3-15% of NASH patients will develop cirrhosis and liver failure (Ascha, et al., 2010). Hepatic disease state is considered a primary factor in the altered disposition of many drugs (Lucena, et al., 2003). NAFLD and particularly, the pathophysiological stage of NASH is a relevant concern to health-care professionals because of the associated risk of ADRs that may accompany drug administration. The objective of the current study is to comprehensively analyze gene expression alterations of ADME genes in the progression of human NAFLD. The results demonstrate a coordinate regulation at the global level resulting in an enrichment of downregulated uptake transporter genes in NASH. DME and efflux transport categories did not exhibit any enrichment for either up or downregulation (Figure 3).

Previous studies have examined gene expression changes in human NAFLD livers classified as steatotic (Greco, et al., 2008) and in livers classified as NASH using a variety of microarray platforms (Baranova, et al., 2007;Rubio, et al., 2007;Younossi, et al., 2005a). These studies confirm that liver disease status alters gene expression changes. The microarray data presented here for the three separate pathological classifications (normal, steatosis, and NASH) reveal statistically significant global gene

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expression alterations with the majority of expression changes occuring in the pathological transition from steatosis to NASH and not from normal to steatosis (Figure 2, Table 1). This data confirms previous studies that show an accumulation of gene expression changes in NASH (Rubio, et al., 2007). The results we present in the current study comprehensively analyze ADME gene expression alterations across each of the stages of human NAFLD and demonstrate that progression to NASH with its accompanying features of the second hit, alters this critical category of genes. The limited number of human samples in this study demonstrated large variances in the ADME gene expression as observed in the PCA between samples diagnosed as steatotic and those diagnosed as NASH (Figure 5). Despite the limited size of the sample groups we show significant expression changes in ADME genes, specifically the transporters in the steatosis versus NASH and normal versus NASH comparisons. Other studies have reported transporter expression changes in patients with inflammatory disease states such as hepatitis and alcoholic liver disease (Morgan, 2001;Renton, 2005). More recently, our laboratory reported gene expression changes of cytochrome P450 enzymes in human NAFLD liver samples (Fisher, et al., 2009c). Changes in DME expression may be important since as many as 75% of all drugs are biotransformed by the P450 enzymes alone in humans (Guengerich, 2008).

The pairwise comparison analysis for the DME gene category reflect upon findings of previous studies done in our laboratory that examined P450 enzyme activity and changes at the transcriptional and translational levels in this same set of human samples. Selected P450s demonstrated significant decreasing trends in mRNA levels

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with disease progression (Fisher, et al., 2009c). Another study conducted by our laboratory demonstrated that the phase II conjugating glutathione transferase (GSTs) phase II enzymes exhibited an increasing trend in mRNA expression with progression of human NAFLD (Hardwick, et al., 2010). The diversity in differential gene regulation within the DME category is clearly evident from these studies.

The pairwise comparison analysis of 515 transporter genes shows the distinctive roles of transporters in either efflux or uptake (Figure 2 & 3). The uptake transporter genes revealed a significant over-representation for genes downregulated for both NASH pairwise comparisons (Figure 3). These results extend previous findings of our laboratory in a rodent NAFLD model to humans. Downregulation of OATP transporter gene expression in the rodent NAFLD model parallels the downregulated uptake transporter expression seen in the current human microarray data (Fisher, et al., 2009a). In that study, rat Oatp1a1, Oatp1a4, Oatp1b2 and Oatp2b1 mRNA expression levels were significantly decreased in the methionine and choline deficient diet rodent model of NASH, leading to a functional impairment in the uptake and subsequent elimination of bromosulphothalein. Based on the similarity in decreased uptake transporter expression, the current data implies a similar functional impairment of the uptake transport process in human NASH (Figure 3).

The over-representation of uptake transporter genes downregulated (Figure 3) suggests the presence of a coordinate transcriptional regulation in humans with NASH. Specifically, downregulation of multiple uptake transporters could prevent the

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accumulation of xenobiotics and toxic intermediates in a diseased liver already compromised by oxidative stress. Investigations have revealed other expression alterations in hepatobiliary transporters in mouse models administered toxic doses of acetaminophen and carbon tetrachloride (Aleksunes, et al., 2005). Coordinate gene expression regulation of ABCC efflux transporters in these mouse models contributed to a reduced chemical burden and hepatoprotection (Aleksunes, et al., 2006).

Multiple mechanisms for a coordinated gene expression response have been identified in the altered regulation of key ADME genes (Kohle and Bock, 2009). Multiple phase I and II DMEs and transporters are coordinately regulated by nuclear receptors and transcription factors. This integrated biotransformation system includes such components as the aryl hydrocarbon receptor (AHR), constitutive androstane receptor (CAR), liver X receptor (LXR) and nuclear factor erythroid 2-related factor transcription factor (Nrf2). Studies of the phase I P450 DMEs, phase II conjugating enzymes, efflux and uptake transporters in our laboratory support the theme of a coordinate regulation in progressive NAFLD (Fisher, et al., 2008;Fisher, et al., 2009a;Fisher, et al., 2009c;Hardwick, et al., 2010;Lickteig, et al., 2007a).

The remodeling of ADME gene expression in the progression of NAFLD is an important consideration in the diagnosis. The pathological stage of steatosis in our small sampling of human liver samples did not demonstrate significant expression changes from that of normal in this study. Therefore the pathological staging of NAFLD is critical in identifying patients with alterations in the expression of ADME genes. The ADME gene expression

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changes we have presented in this study reveal an important downregulatory function of uptake transport genes. While this coordinated downregulation of the ADME gene category is indicative of a hepatoprotective response, these findings may also have implications in drug dosing regimens. These implications should be taken into account by health care practitioners and pharmaceutical investigators when making decisions regarding pharmacotherapy for the NAFLD patient population.

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Authorship Contribution

Participated in research design: Lake, Billheimer, Klimecki and Cherrington.

Conducted experiments: Lake and Hardwick.

Contributed new reagents or analytic tools: Fisher, Jackson, Billheimer and Klimecki.

Performed data analysis: Novak, Lake and Klimecki.

Wrote or contributed to the writing of the manuscript: Lake, Novak, Hardwick, Billheimer, Klimecki and Cherrington.

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Footnotes.

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Figure Legends.

Figure 1. Principal component analysis of global gene expression. The first and second principal components used in this graphical representation account for 22% and 12% respectively of the total variance in the global gene expression. The PCA plot determines the factor that contributes to the variance. Sex and age do not appear to cause systematic changes in gene expression.

Figure 2. Differential gene expression in progressive human NAFLD. Venn diagrams summarize the magnitudes of genes differentially expressed for the global, DME, and transporter gene categories. Each circle represents one of the three pairwise comparisons between disease states (normal versus steatosis, steatosis versus NASH, and normal versus NASH).

Figure 3. Distribution histograms of gene expression. Up and downregulated gene expression is represented by red and green respectively in the barplots and distribution histograms for each of the three pairwise comparisons: normal versus steatosis, steatosis versus NASH, and normal versus NASH. Global gene expression changes are illustrated in the large barplot for All Genes. Gene expression changes for DME, all transporters, efflux only, and uptake only transporter gene categories are represented by smaller barplots and distribution histograms. The vertical black bar in the distribution histograms represents the actual number of gene expression changes observed for a gene category as tested against an expected distribution of randomly chosen genes. N-Normal, Ste-Steatosis, NSH-NASH. Significance is determined by $p < 0.05^{*}$.

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Figure 4. Hierarchical clustering. Heat maps were generated to represent hierarchical clustering data of differential gene expression for each gene category. Colored boxes represent upregulation (red) and downregulation (green). Tree-structures matched to the two axes represent patterns between diagnosis groups and gene expression differences. Genes are represented on the x axis and specimen diagnosis on the y axis (normal-yellow, steatosis-orange, NASH-red).

Figure 5. Principal component analysis of ADME gene expression. Principal component analysis of the categories for the DMEs, all transporters, uptake only, and efflux only transporters are shown. The first two principal components representing the majority of the variance in the gene data are plotted to show similarities or differences in the expression changes for normal, steatosis and NASH specimens.

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Table 1.

Gene Set	Normal vs Steatosis	Steatosis vs NASH	Normal vs NASH
All Genes	1277 (3.8%)	5746 (17.3%)	9724 (29.2%)
DMEs	3 (1.2%)	57 (22.1%)	81 (31.3%)
Transporters	25 (4.8%)	150 (29.1%)	210 (40.8%)

Table 1. The number and percentage of differentially expressed genes for each gene category is calculated from 33,252 global genes, 258 DME genes, and 515 transporter genes.

FIGURE 1

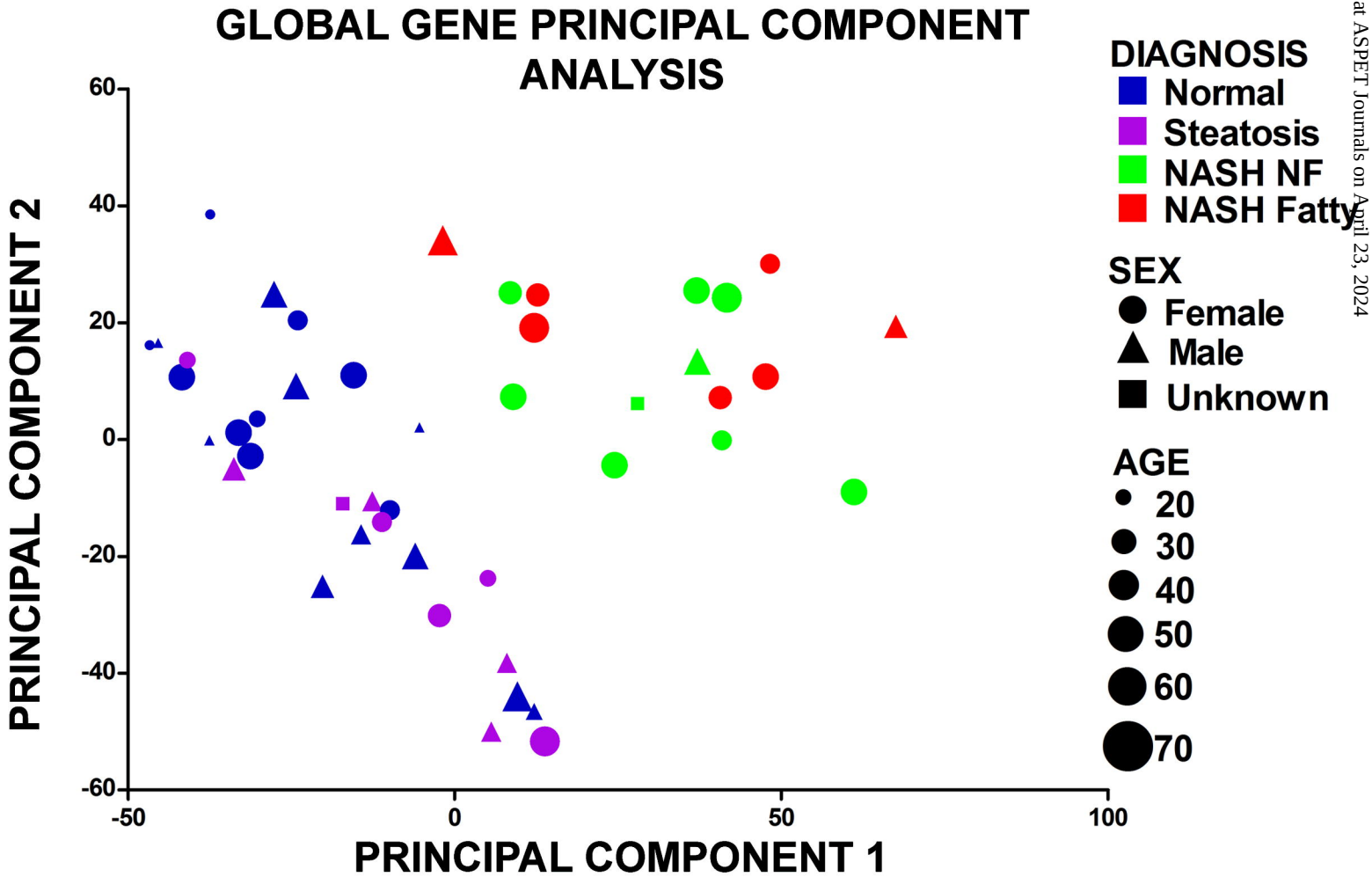

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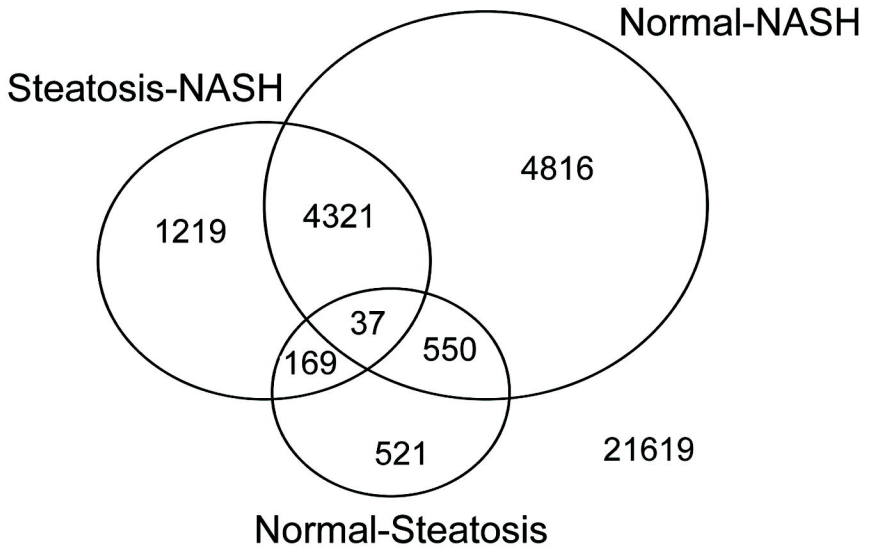
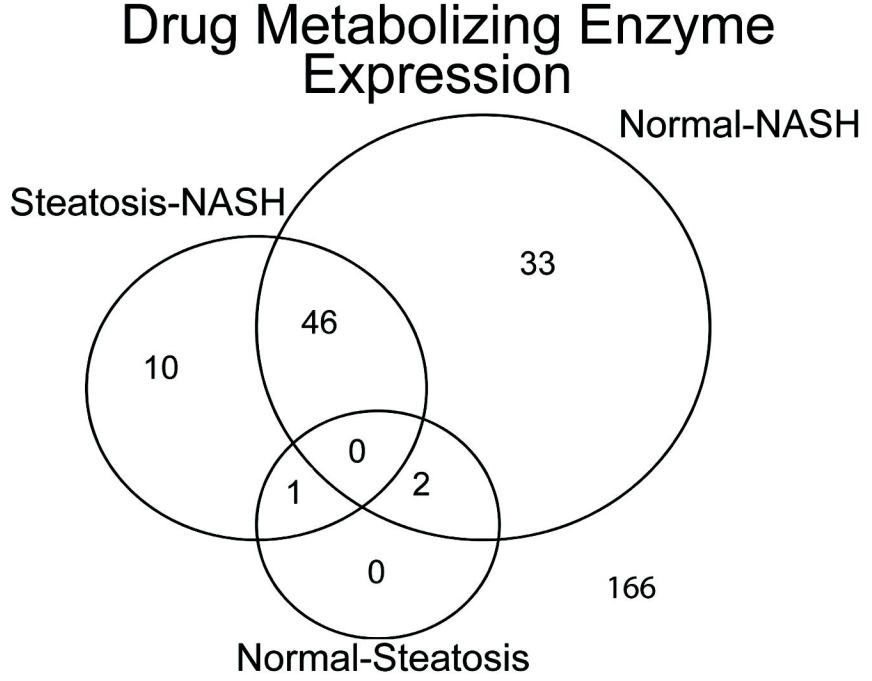
FIGURE 2





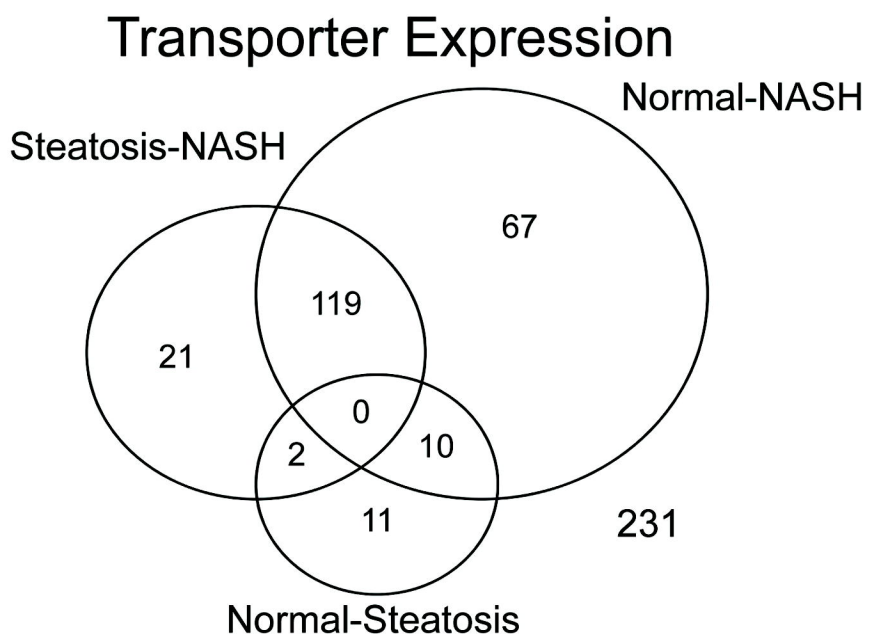


FIGURE 3

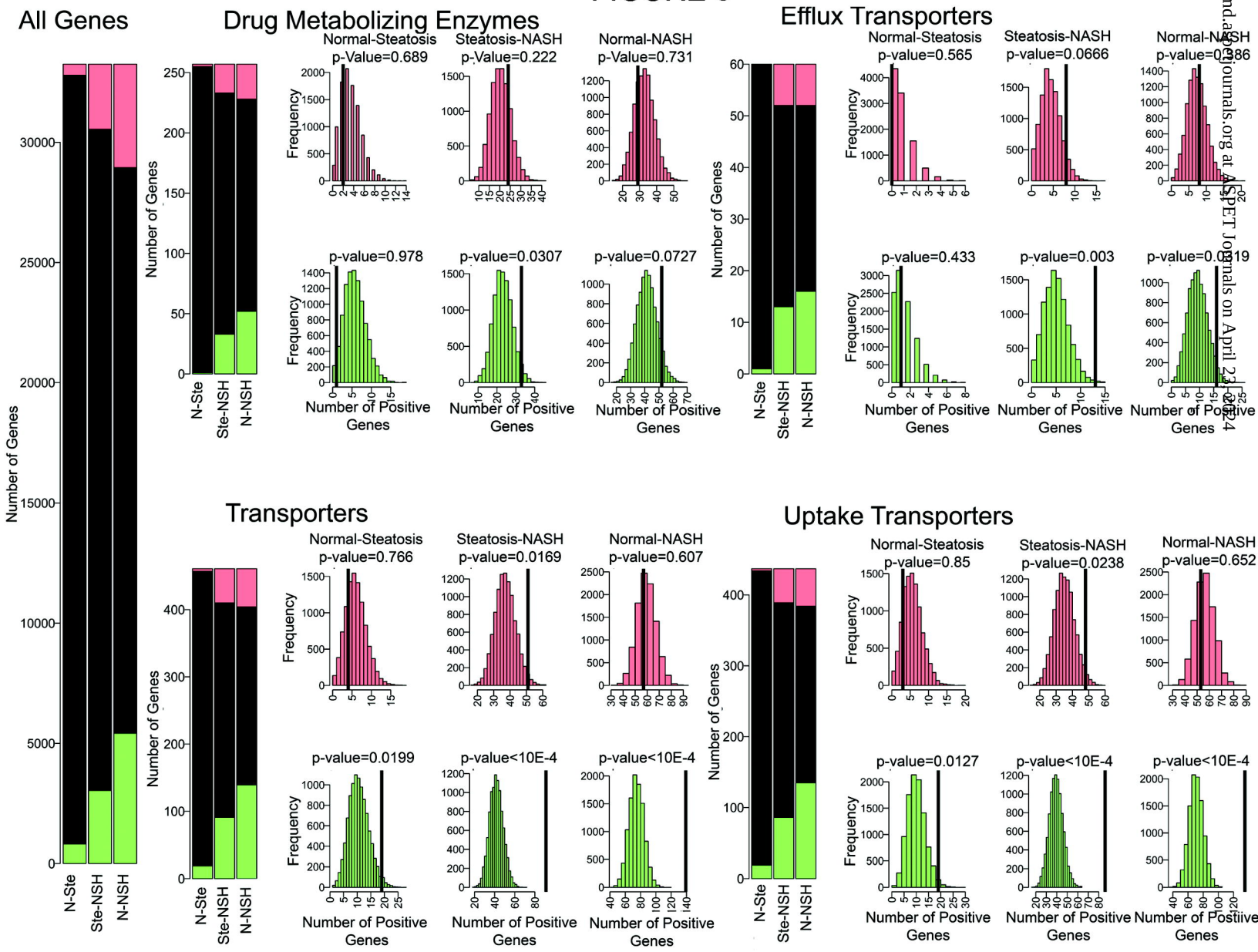


FIGURE 4

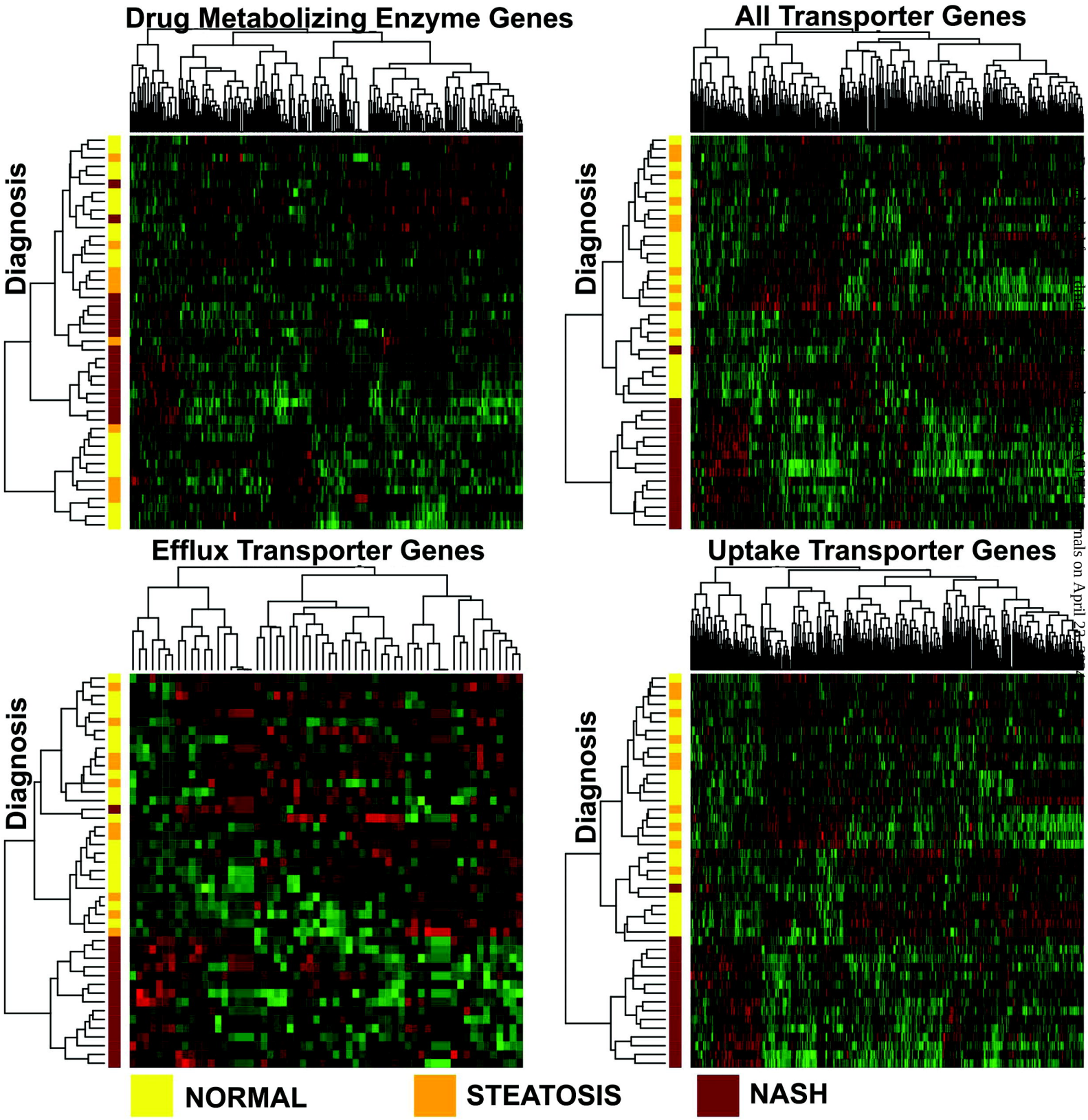

Drug Metabolizing Enzyme Genes
All Transporter Genes
Efflux Transporter Genes
Uptake Transporter Genes
Diagnosis
NORMAL
STEATOSIS
NASH

FIGURE 5