CHARACTERIZATION OF THE ONTOGENY AND INTER-INDIVIDUAL VARIATION OF GENES IN CHOLESTEROL AND STATIN PATHWAYS

By

Stacy Cassat

Submitted to the graduate degree program in Clinical Research and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

________________________________
Chairperson Brooke Fridley, PhD

________________________________
J. Steven Leeder, PhD, PharmD

________________________________
Jonathan B. Wagner, DO

________________________________
Lane Christenson, PhD

Date Defended: April 28, 2015
The Thesis Committee for Stacy Cassat
certifies that this is the approved version of the following thesis:

CHARACTERIZATION OF THE ONTOGENY AND INTER-INDIVIDUAL VARIATION OF
GENES IN CHOLESTEROL AND STATIN PATHWAYS

Chairperson, Brooke Fridley, PhD

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Abstract

Introduction: Previous studies have shown that gene expression can change throughout development,¹ and therefore genotype-phenotype associations found in adults might not be observed in children of all ages. The purpose of this study was to 1) characterize the ontogeny of 30 genes in pathways related to cholesterol synthesis and/or statin action or toxicity in pediatric liver samples, and 2) assess the in vitro and in vivo consequences in children of genetic variation in 3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase (HMGCR) and heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), two genes implicated in altered cholesterol levels and/or statin response.²,³

Methods: RNA and DNA were isolated from pediatric liver samples (n=62), and DNA was isolated from patients in the Cardiology Pharmacogenomic Repository (CPR) (n=195). For Aim 1, the ontogeny of mRNA expression from 30 genes related to cholesterol and statin metabolism as measured by RNA-seq in the liver samples was assessed. For Aim 2, all postnatal samples were genotyped for rs1920045 (HNRNPA1) and rs3846662 (HMGCR), and genotypes were tested for association with either alternative splicing in vitro (liver samples) or plasma lipid levels in vivo (CPR). Statistical analyses on liver sample data were conducted with Kruskal-Wallis or Wilcoxon tests with Bonferroni correction. Analysis of CPR samples was completed with ANOVA, Kruskal-Wallis or Wilcoxon tests with Bonferroni correction. Samples were stratified by race and analyses were repeated. Liver sample use was deemed non-human subjects research and the CPR was approved by the Pediatric IRB.

Results: Analysis of postnatal liver samples revealed age-related changes in Ensembl-based total and primary mRNA transcript expression of ABCB1 (p<0.0008). Inclusion of prenatal samples revealed 13 additional genes with age-related expression changes during development in either
the Ensembl or UCSC based data. The ratios of alternative to canonical transcripts of \textit{HMGCR} trended towards significance in the \textit{HMGCR} and \textit{HNRNPA1} dominant genotype models (p=0.0465, 0.0470 respectively). CPR analysis suggested a relationship between \textit{HMGCR} genotype and low-density lipoprotein cholesterol (LDL-C) or total cholesterol (TC) in African Americans, or \textit{HNRNPA1} genotype and TC in Caucasians, but these relationships did not achieve statistical significance.

**Conclusion:** Although trends in age-related changes in gene expression, and genotype-phenotype associations, were observed for several genes of interest, the number of statistically significant associations was limited by use of a stringent criterion for multiple testing as well as intra-group variability and relatively small sample sizes given the amount of variability observed. Until these issues are resolved in a larger number of samples, it is premature to conclude genotype-phenotype associations observed in adults will also be present in children at all ages.
Acknowledgements

I would like to acknowledge Children’s Mercy Hospital Kansas City for providing me with the resources to complete this project. Thank you to my mentor, Dr. Steve Leeder, who provided me with knowledge, encouragement, and guidance throughout the year. Additionally, thank you to Dr. Jon Wagner, who brought me into the statin projects and mentored me through the clinical aspects of the research. Thank you to Dr. Roger Gaedigk, who taught me the essential components of lab work, helped me with experimental design and mentored me in research analysis and presentation. Thank you to Dr. Chengpeng “Charlie” Bi and Dr. Shui Quing Ye for their RNA-seq work, and for letting me use this data for the project. Thank you to the Clinical Pharmacology Research Assistants – Amber, Annie, Emily, Erika, and Mandy – for sharing your lab space, time and knowledge with me throughout the year.

I would also like to acknowledge the University of Kansas Medical Center for providing me with formal research education and funding for the program. Dr. Lane Christenson generously shared lab equipment and materials, as well as experimental design input. Dr. Brooke Fridley, the chair of this thesis committee, was essential in solidifying the analytical portion of the project and provided key insight into RNA-seq analysis to ensure that this thesis was analytically thorough.
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INTRODUCTION

Between childhood and early adulthood, significant precursors to cardiovascular disease (CVD), begin forming in coronary arteries. The Bogalusa Heart Study found that the presence of fatty streaks in coronary arteries increased in prevalence from 50% at 2 – 15 years of age to 85% at 21 – 39 years of age, and the presence of raised fibrous plaques increased in prevalence from 8% to 69% during this time. Additionally, the emerging childhood obesity epidemic has accelerated the concern over CVD risk factor development among children.

In response, the National Heart, Lung, and Blood Institute (NHLBI) released updated pediatric cardiovascular health guidelines in 2011, which recommended universal lipid screening for children between nine and 11 years of age. Lipid lowering therapy during adolescence is one focus of the updated guidelines, and statins are recommended as a first-line pharmacotherapeutic option for children with elevated lipid levels. Statins constitute a medication class that acts primarily by inhibiting 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) in the liver, which is the rate-limiting enzyme in endogenous cholesterol synthesis. Recently, it was estimated that over 200,000 children qualify for statin use in the U.S.

There are presently seven FDA-approved drugs within the statin class: atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. Transporters are involved in the import of some statins into hepatocytes. Once in the hepatocyte, statins exert their clinical effect on the cholesterol pathway, can be transformed by multiple enzymes into active or inactive forms, and are later excreted into the bile by transporter proteins. Due to the well-defined pathways involved in their pharmokinetics and pharmacodynamics, statins offer a unique and important opportunity to explore genetic ontogeny and variation involved with their disposition and molecular effect as it relates to clinical response.
Within the medical community, there exists some concern over disrupting cholesterol metabolism during childhood development.12 Lipids and sterols are involved in neuronal development as well as synthesis of steroid hormones, key components of development.12 Statins have been shown to be safe and efficacious in pediatric populations; however, much of this data comes from studies with relatively short follow-up periods.13 Considering how statins are commonly used long-term, which could equate to decades if initiated in childhood, the safety of statin use during childhood and adolescence has yet to be fully determined.13 Initial studies have shown conflicting results surrounding the impact of statins on hormone levels, and neuronal effects, if any, may not be apparent until much later on in life.12,13 A study to fully address these concerns would likely be resource and time intensive. Surrogate endpoints, such as lipid levels and liver enzymes, are currently used to assess both the efficacy and safety of statins in children.12 Additional information could be gained from characterizing the genetic background of statin therapeutic and metabolic pathways during development.

It is widely recognized that gene expression changes as children grow and develop;1 however, the molecular-level details of this process for many genes involved in statin pharmacokinetics and pharmacodynamics are largely unknown. Studies on some of the most common drug metabolizing enzymes, cytochrome P450 (CYP) enzymes, have shown that the expression of these genes changes throughout childhood development.14 This suggests that the expression ontogeny of other drug metabolizing and transporting genes could also be dynamic and thus greatly affecting pharmacokinetics and pharmacodynamics in pediatric patients. Although the ontogeny of CYP enzymes has been extensively characterized, targets of statin action remain ontogenetically undescribed.
Additionally, multiple genes have been identified that may contribute to variability in statin response.\textsuperscript{15} For instance, some statins or statin metabolites have been shown to be substrates of a drug transporter, MDR1, encoded by the gene \textit{ABCB1},\textsuperscript{16} and variation within this gene has shown some association with response to treatment with particular statins;\textsuperscript{17,18} however, the effect may not be clinically significant.\textsuperscript{17} Our research team and a collaboration in the Netherlands have published on its hepatic ontogeny in prenatal, neonatal and a small group of pediatrics, yet \textit{ABCB1}’s hepatic expression at different points in childhood was not fully investigated.\textsuperscript{19} An additional gene, \textit{SLCO1B1}, codes for a transporter thought to import statins into the hepatocyte.\textsuperscript{20} In adults, genetic variation in this gene has been associated with increased plasma statin levels and clinical myopathy, yet in children it was shown to be associated with decreased plasma drug levels.\textsuperscript{21,22} Furthermore, Gryn and Hegel published a review describing genetic variation in dozens of genes, such as \textit{HMGCR} and \textit{COQ2}, which have been associated with statin therapy in adults\textsuperscript{15} and has yet to be fully investigated in the pediatric setting.

Recently, an alternative transcript of the statin target, \textit{HMGCR}, that lacks exon 13 (\textit{HMGCR}\textbf{Δ}13) has gained attention for its association with reduced response to statins in adults.\textsuperscript{3} A variant in \textit{HMGCR}, rs3846662, was associated with increased expression of \textit{HMGCR}\textbf{Δ}13 relative to the expression of the canonical \textit{HMGCR} transcript including exon 13 (\textit{HMGCR}ex13) in Caucasian lymphoblastoid cell lines (LCLs).\textsuperscript{23} \textit{HMGCR} has a binding motif for heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) in the intronic region between exons 13 and 14 (Figure 1).\textsuperscript{2} \textit{HNRNPA1} has been shown to impact the alternative expression of \textit{HMGCR}; however the relationship was not investigated in the dynamic metabolisms of children.\textsuperscript{2} Additionally, it was shown that a variant upstream of \textit{HNRNPA1}, rs1920045, was associated with altered expression of an alternative transcript of \textit{HNRNPA1} that included exon-8.
(HNRNPA1ex8) versus the canonical transcript that excluded exon 8 (HNRNPA1Δ8) (Yu, et al., Supplemental Figure S6-A). This splice variant, HNRNPA1ex8, in turn was hypothesized to increase expression of HMGCRΔ13. It is therefore hypothesized that ‘T/T’ genotype will alter relative HMGCRΔ13 expression. The transcript of HMGCRΔ13 may have clinical significance, as a relative increase in expression of the transcript has been associated with cholesterol-lowering in response to statins, and SNP rs3846662 linked to this expression is associated with lipid levels in adults. 

**A. HMGCR Alternative Splicing**

**B. HNRNPA1 Alternative Splicing**

Figure 1. Canonical and Alternative Splicing of HMGCR (A) and HNRNPA1 (B). Solid lines indicate the splicing pattern for the canonical transcript. Dashed lines indicate the splicing pattern for the alternative transcript.²

Phenotypic variation due to genetic differences that have been observed in adults may only be apparent in children once gene pathways are fully developed. In order to determine this, the developmental trajectory of gene expression must be described. Additionally, genetic variants with known phenotypes in adults need to be studied in children to discern whether the phenotype-genotype associations remain consistent. The objectives of this study were to (1) characterize the ontogeny of mRNA expression in pediatric liver samples for a set of 30 genes in pathways associated with cholesterol synthesis and/or statin therapeutic response or toxicity, and
(2) investigate the consequences of genetic variation in \textit{HMGCR} and \textit{HNRNPA1} in a pediatric context, both \textit{in vitro} and \textit{in vivo}.

\section*{METHODS}

\subsection*{Tissue Repository}

Human liver tissues were obtained from two National Institute of Child Health and Development supported tissue repositories – the University of Maryland Brain and Tissue Bank for Developmental Disorders (Baltimore, MD) and the Laboratory of Human Development at the University of Washington (Seattle, WA), the Liver Tissue Cell Distribution System, and Xenotech, LLC (Lenexa, KS). The age of removal of liver samples ranged from prenatal (103 days post-conception) to late adolescence (17 years). All individuals were deceased at the time of removal and the causes of death varied. The use of this repository has been deemed non-human subjects research by the Children’s Mercy Hospital (CMH) Pediatric Institutional Review Board (IRB). Characteristics of the samples used from the liver repository are described in Table 1. RNA and DNA were extracted from liver tissues with the Qiagen AllPrep DNA/RNA Mini Kit or the Qiagen AllPrep DNA/RNA/miRNA Universal Kit (Hilden, Germany) and stored at -80°C and 4°C, respectively.

RNA samples for RNA-sequencing (RNA-seq) were selected based on the absence of documented liver disease or medications affecting the liver, representative distribution across developmental ages, and an RNA Quality Index (RQI) above 3. RQI is a quality measure, analogous to RNA Integrity Number (RIN) from the Agilent Bioanalyzer, on a scale from zero to 10, with 10 being considered the highest quality. The RNA was run in a microfluidic StdSens chip on an Experion\textsuperscript{TM} (Bio-Rad, Hercules, CA).
Table 1. Characteristics of Liver Tissue Samples (n=61).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N</th>
</tr>
</thead>
<tbody>
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<td>Group 0 (prenatal)</td>
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<tr>
<td>Group 1 (birth to &lt;1 year)</td>
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<tr>
<td>Group 2 (1 to ≤6 years)</td>
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<td>Group 3 (&gt;6 to ≤12 years)</td>
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<tr>
<td>Group 4 (&gt;12 years)</td>
<td>8</td>
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<tr>
<td>Sex</td>
<td></td>
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<td>Male</td>
<td>45</td>
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<tr>
<td>Female</td>
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<tr>
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<td>Caucasian</td>
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<td>African American</td>
<td>8</td>
</tr>
<tr>
<td>Other, Multiple, Unknown</td>
<td>35</td>
</tr>
</tbody>
</table>

Living Repository – Cardiology Pharmacogenomics Repository (CPR)

The Cardiology Pharmacogenomics Repository (CPR) is a living patient repository with DNA isolated from biospecimens (i.e. saliva, blood) of each participant, using the Sigma GeneElute™ Mammalian Genomic DNA Miniprep Kit (St. Louis, MO) or QIAamp® DNA Blood Mini Kit (250) (Hilden, Germany) according to manufacturer protocols. The enrollment criteria include: age 0 to 26 years, patients with existing cardiac disease, patients at risk of CVD (i.e. abnormal lipid profile, obesity, diabetes mellitus, family history of cardiac disease), and/or patients receiving a cardiovascular pharmacotherapeutic. Participants were recruited from the nutrition, weight management, general cardiology, and preventative cardiology clinics at CMH. The samples were coded to protect patient privacy but could be linked back to participants’ Electronic Medical Records (EMR) by the Principal Investigator for retrospective chart review. All samples in the CPR as of February 25, 2015 (n = 195) were included in this study, except for one participant who had missing data on all measurements. A total of eight patients were
subsequently excluded: five patients who had been enrolled following cardiac transplantation and three who had no lipid panel documented in their EMRs prior to statin therapy (final n=186). The CPR was approved by the CMH Pediatric IRB.

**CPR Clinical Data Collection**

Retrospective chart review was conducted to collect values for total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, height, weight, body mass index (BMI), age (in years and months) at time of lab draw, and sex for each CPR participant. Information regarding documented lipid-lowering medication use at the time of lab draw was also collected. Lipid values were taken from the most recently documented lab draw up to and including the intervention visit, except one participant, whose lipid values after the visit were used. The BMI was taken from the intervention visit documentation. To get the correct age from the data for BMI percentile calculation, the month difference between the lipid panel date and the visit date was calculated and then added to the age at time of the lab. There were two participants for which this calculation could not be completed. This calculated age was then used for calculation of the BMI percentile. Race information was taken from documentation of the participant’s self-reported race at the time of his or her enrollment. Races were inclusive of those identifying with a Hispanic ethnicity for analysis (i.e. Caucasian race with non-Hispanic ethnicity was included with Caucasian race with Hispanic ethnicity, and the same was done for ethnically Hispanic African Americans).

Characteristics of the CPR participants are described in **Table 2**. The collection of this information was performed with the CPR Principal Investigator, Dr. Jon Wagner, to ensure the most appropriate dates were selected and the correct information was recorded.
<table>
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**Clinical Characteristics (NHLBI “Acceptable Concentration”)*6**

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<tr>
<td>Age, years</td>
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<tr>
<td>Baseline Total Cholesterol, mg/dL (&lt;170 mg/dL)</td>
<td>242 (52)</td>
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<td>Baseline LDL Cholesterol, mg/dL (&lt;110 mg/dL)</td>
<td>166 (52)</td>
</tr>
<tr>
<td>Baseline HDL Cholesterol, mg/dL (&gt;45 mg/dL)</td>
<td>47 (13)</td>
</tr>
<tr>
<td>Baseline Triglycerides, mg/dL (Age 0 to 9 yrs, &lt;75 mg/dL)</td>
<td>151 (81)</td>
</tr>
<tr>
<td>Age 10 to 19 yrs &lt;90 mg/dL)</td>
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**RNA-seq and Bioinformatics**

RNA-seq was completed for 62 liver tissue samples from the repository. One μg total human liver RNA was used to generate TrueSeq Ribo-Zero libraries with the Illumina TruSeq Stranded Total RNA with Ribo-Zero Globin Library Prep Kit. Samples were run on the Illumina
HiSeq 1500 (Genomics Research Core Lab) with paired-end (2 x101), deep sequencing coverage (104x).

RNA-seq reads were output in FASTQ format, and the data were organized by individual samples. Quality control was performed with FastQC. No sequence trimming or removal was performed. Two reference transcriptomes were prepared, one from the European Molecular Biology Laboratory (Ensembl) database and one from the University of California Santa Cruz (UCSC) genome database. RNA-seq Expectation Maximization (RSEM) was performed with Bowtie2 to index the reference transcriptomes and align the sample reads. Based on the maximum likelihood model, RSEM was run to assign reads to annotated transcripts and estimate the abundance of each transcript. In data analysis, one sample was removed as a library size outlier.

Genes of interest for this study were identified from statin pharmacodynamic and pharmacokinetic pathways (PharmGKB),\textsuperscript{10,11} reviews of the pharmacogenomics of statin disposition and response,\textsuperscript{15,25,26} and genome-wide association studies of variability in cholesterol levels (for example Tikkanen, \textit{et al.} and citations therein).\textsuperscript{27} The query included total and primary transcripts of \textit{ABCB1, AGTR1, AMPD1, APOC1, APOC2, APOE, ATP2B1, CETP, CLMN, CPT2, COQ2, CYP7A1, DMPK, DNAJC5B, GATM, HMGCR, HNRNPA1, HTR3B, HTR7, KIF6, LDLR, LPA, LPIN1, MYLIP, NOS3, PCSK9, PYGM, RYR1, SLC10A1,} and \textit{SLCO1B1}. This set of genes was analyzed for total and primary transcript mRNA expression changes associated with age as a categorical variable, from prenatal (103 days post-conception) to age 17 years.

Additionally, the RNA-seq dataset was queried for mRNA expression changes associated with \textit{HMGCR} rs3846662G>A and \textit{HNRNPA1} rs1920045C>T. The transcripts of interest were \textit{HMGCR} transcripts including and excluding exon 13 (ENST00000287936 and
ENST00000343975, respectively) and HNRNPA1 transcripts including and excluding exon 8 (ENST00000340913 and ENST00000547276, respectively). This SNP-associated gene expression had been previously described in LCLs.\textsuperscript{2,23}

**Genotyping**

All postnatal DNA samples (liver and CPR) in this study were genotyped for HMGCR rs3846662G>A and HNRNPA1 rs1920045C>T using TaqMan\textsuperscript{®} SNP genotyping assays C\_2838669\_10 and C\_12057681\_10, respectively (LifeTechnologies, Carlsbad, CA), with KAPA Probe Fast qPCR Master Mix (2x) ABI Prism\textsuperscript{®} (KAPA Biosystems, Boston, MA) on an AB 7900HT Fast Real-Time PCR System (Applied Biosystems). For each reaction, 3 to 19 ng of DNA were used, in a total volume of 6 uL, with the exception of one sample that amplified with less than 0.53 ng of DNA in this volume. If amplification of a sample failed, the input DNA was doubled. The cycling conditions for both assays were 95°C, 2 min, [95°C, 10 sec, 60°C, 60 sec] for 45 cycles. DNA samples from the Coriell Institute for Medical Research were used as controls (HMGCR, G/G: NA17294, HMGCR, G/A: NA12813, HMGCR, A/A: NA12877; HNRNPA1, C/C: NA12882, HNRNPA1, C/T: NA17204, HNRNPA1, T/T: NA06989). Ten percent of samples were randomly selected and run a second time to check for consistency. For each marker, call rates were computed (100\%) along with Hardy-Weinberg Equilibrium (HWE) tests by race (Table 3).

<table>
<thead>
<tr>
<th>Gene</th>
<th>RsID</th>
<th>Cohort</th>
<th>Position</th>
<th>Alleles</th>
<th>Total Genotype, ( N )</th>
<th>Cau. Genotype, ( N )</th>
<th>Af. Am. Genotype, ( N )</th>
<th>HWE p-value</th>
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</table>
**Statistical Analysis**

Association of age with gene expression measured by RNA-seq for the 30 genes of interest was carried out using Kruskal-Wallis tests (i.e., non-parametric ANOVA) with Bonferroni correction (significance: p<0.0016). Age was treated as a categorical variable and gene expression - in transcripts per million reads (TPM) - was continuous. Age groups were based on developmental stages (Group 0: prenatal, Group 1: birth to <1 year, Group 2: 1 to ≤6 years, Group 3: >6 to ≤12 years, and Group 4: >12 years). Categorical age group analysis was selected over continuous age analysis to allow detection of non-linear expression changes that may occur during development. Gene expression was evaluated as total transcript expression and primary transcript expression. The most abundant transcript in the RNA-seq output was chosen as the primary transcript. The significance threshold with Bonferroni correction was higher for the primary transcript analysis because only 27 genes had more than one transcript from which to choose a primary transcript in the RNA-seq output for the Ensembl dataset and only 13 genes had more than one transcript in the UCSC dataset (p<0.0018 and p<0.0038, respectively). Separate analyses were performed including and excluding the prenatal samples. Pairwise comparisons were performed with Tukey’s HSD or Steel-Dwass tests.

The ratios of alternative to canonical transcript expression for **HMGCR** and **HNRNPA1** were calculated from the measured RNA-seq transcript data and log-transformed. Associations of log-transformed ratios with the genotypes of interest were assessed using Wilcoxon (chi-square approximation to the one-way test) or Kruskal-Wallis tests with Bonferroni correction. Both general genetic (co-dominant) and dominant genetic models were performed, and ratios of gene expression were viewed as continuous variables. The samples were stratified by race and all
analyses were repeated for the Caucasian subgroup. There was an insufficient number of African American samples to perform separate analyses for this subgroup.

Associations between the genotypes (\textit{HMGCR} or \textit{HNRNPA1}) and lipid levels (TC, LDL-C, HDL-C, or triglycerides) were evaluated using ANOVA with log-transformed lipid levels and genotype modeled with a general genetic (co-dominant) model. BMI percentiles were calculated from the raw BMI, sex, and age at BMI visit. These percentiles were categorized into underweight (<5\textsuperscript{th} percentile), normal (5\textsuperscript{th} to <85\textsuperscript{th} percentile), overweight (85\textsuperscript{th} to <95\textsuperscript{th} percentile), or obese (≥95\textsuperscript{th} percentile) according to the guidelines from the Center for Disease Control (CDC). One participant was over 20 years old, and thus was classified according to the adult CDC guidelines. BMI was then analyzed as a categorical variable with a contingency table and Chi-square or Fisher’s Exact test. Due to fewer than 3 participants being classified as underweight, this category was excluded from the BMI analysis. Samples were stratified by race and all CPR analyses were repeated for Caucasians and African Americans separately using Kruskal-Wallis or Wilcoxon tests with Bonferroni correction.

All statistical analysis was conducted in JMP\textsuperscript{®}, Version 10. SAS Institute Inc., Cary, NC, 1989-2007 or R version 3.1.2 (www.R-project.com).\textsuperscript{28}

RESULTS

Hepatic Ontogeny

The Ensembl-based total gene expression ontogeny analysis of the gene set including prenatal samples revealed 12 genes with expression changes significantly associated with age: \textit{ABCB1} (p<0.0001), \textit{APOC1} (p=0.0008), \textit{ATP2B1} (p<0.0001), \textit{CETP} (p<0.0001), \textit{COQ2} (p=0.0003), \textit{CYP7A1} (p<0.0001), \textit{DMPK} (p<0.0001), \textit{HMGCR} (p=0.0002), \textit{HNRNPA1} (p<0.0001), \textit{KIF6} (p<0.0001), \textit{MYLIP} (p<0.0001), and \textit{RYR1} (p=0.0005) (Table 4). In pairwise
comparisons for each of these genes, the expression in Group 0 (prenatal) differed from the expression in at least one other group (Table B1). Analysis of the transcripts aligned to the UCSC database showed significant age associations in the same genes as in the Ensembl analysis, except for COQ2 (p=0.0059) (Table 4). Two additional genes were significant in the UCSC-based analysis: APOC2 (p=0.0003) and LPIN1 (p<0.0001). In pairwise comparisons for these two genes, the expression in Group 0 (prenatal) differed from the expression in at least one other group (Table B1).

The Ensembl-based primary transcript expression analysis including prenatal samples revealed significant associations between age and the expression of ABCB1 (p<0.0001), APOC1 (p=0.0008), CETP (p=0.0002), CLMN (p=0.0003), COQ2 (p=0.0002), DMPK (p=0.0001), HMGCR (p=0.0001), HNRNPA1 (p=0.0002), KIF6 (p=0.0005), LPIN1 (p=0.0002), and MYLIP (p<0.0001) (Table 5). Pairwise analysis showed that Group 0 (prenatal) gene expression differed from the expression in at least one other age group for each of these genes (Table B2). All of these genes that also had primary transcripts in the UCSC-based data showed an analogous relationship between age and primary UCSC transcript expression, with the exception of CETP (p=0.1644) (Table 5).

Due to the different cellular environment of the prenatal liver compared to the postnatal liver, separate analyses were performed excluding the prenatal samples. In the Ensembl-based total gene expression ontogeny analysis of the 30 genes of interest, excluding prenatal samples, only one gene’s expression, ABCB1, showed significant association with age (p=0.0008) (Table 4). There was an increase in ABCB1 expression during development, with Group 1 (<1 year-old) showing significantly different gene expression from other groups (Figure 2; Table 6). In the UCSC alignment, ABCB1 expression trended toward a significant relationship with age but did
not reach significance (p=0.0024) (Figure 3). No other gene’s expression showed a significant relationship with age in the UCSC-based analysis (Table 4).

In the Ensembl-based primary transcript expression ontogeny analysis of the gene set excluding prenatal samples, only \textit{ABCB1} (ENST00000265724) expression was significantly associated with age (p=0.0008) (Table 5). Group 1 (<1 year old) \textit{ABCB1} ENST00000265724 expression differed from other age groups and expression appeared to increase with age (Figure 4; Table 6).
Table 4. Total Gene mRNA Expression Ontogeny Analysis Results.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Kruskal-Wallis p-value Excluding Prenatal</th>
<th>Kruskal-Wallis p-value Including Prenatal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ensembl</td>
<td>UCSC</td>
</tr>
<tr>
<td>ABCB1</td>
<td>0.0008</td>
<td>0.0024</td>
</tr>
<tr>
<td>AGTR1</td>
<td>0.4262</td>
<td>0.2991</td>
</tr>
<tr>
<td>AMPD1</td>
<td>0.4035</td>
<td>0.5261</td>
</tr>
<tr>
<td>APOC1</td>
<td>0.3978</td>
<td>0.2023</td>
</tr>
<tr>
<td>APOC2</td>
<td>0.6872</td>
<td>0.4746</td>
</tr>
<tr>
<td>APOE</td>
<td>0.8336</td>
<td>0.6200</td>
</tr>
<tr>
<td>ATP2B1</td>
<td>0.4890</td>
<td>0.0246</td>
</tr>
<tr>
<td>CETP</td>
<td>0.6463</td>
<td>0.4240</td>
</tr>
<tr>
<td>CLMN</td>
<td>0.9193</td>
<td>0.3689</td>
</tr>
<tr>
<td>COQ2</td>
<td>0.2007</td>
<td>0.0443</td>
</tr>
<tr>
<td>CPT2</td>
<td>0.4767</td>
<td>0.4561</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>0.0041</td>
<td>0.0039</td>
</tr>
<tr>
<td>DMPK</td>
<td>0.0332</td>
<td>0.0072</td>
</tr>
<tr>
<td>DNAJC5B</td>
<td>0.6655</td>
<td>0.1696</td>
</tr>
<tr>
<td>GATM</td>
<td>0.7875</td>
<td>0.7614</td>
</tr>
<tr>
<td>HMGCR</td>
<td>0.9994</td>
<td>0.9632</td>
</tr>
<tr>
<td>HNRNPA1</td>
<td>0.9987</td>
<td>0.7840</td>
</tr>
<tr>
<td>HTR3B</td>
<td>0.8304</td>
<td>0.5911</td>
</tr>
<tr>
<td>HTR7</td>
<td>0.0630</td>
<td>0.0377</td>
</tr>
<tr>
<td>KIF6</td>
<td>0.0043</td>
<td>0.0084</td>
</tr>
<tr>
<td>LDLR</td>
<td>0.5213</td>
<td>0.3911</td>
</tr>
<tr>
<td>LPA</td>
<td>0.8900</td>
<td>0.9933</td>
</tr>
<tr>
<td>LPIN1</td>
<td>0.1658</td>
<td>0.0100</td>
</tr>
<tr>
<td>MYLIP</td>
<td>0.6914</td>
<td>0.3682</td>
</tr>
<tr>
<td>NOS3</td>
<td>0.9440</td>
<td>0.9991</td>
</tr>
<tr>
<td>PCSK9</td>
<td>0.2771</td>
<td>0.1198</td>
</tr>
<tr>
<td>PYGM</td>
<td>0.7973</td>
<td>0.9830</td>
</tr>
<tr>
<td>RYR1</td>
<td>0.0993</td>
<td>0.0494</td>
</tr>
<tr>
<td>SLC10A1</td>
<td>0.2400</td>
<td>0.1682</td>
</tr>
<tr>
<td>SLC10B1</td>
<td>0.6535</td>
<td>0.3704</td>
</tr>
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</table>
### Table 5. Primary Transcript Expression Ontogeny Analysis Results.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Kruskal-Wallis p-value Excluding Prenatal</th>
<th>Kruskal-Wallis p-value Including Prenatal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ensembl</td>
<td>UCSC</td>
</tr>
<tr>
<td>ABCB1 ENST00000265724</td>
<td>0.0008</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AGTR1 ENST00000402260 or NM_032049</td>
<td>0.6082</td>
<td>0.1031</td>
</tr>
<tr>
<td>AMPD1 ENST00000520113</td>
<td>0.6616</td>
<td></td>
</tr>
<tr>
<td>APOC1 ENST00000252491</td>
<td>0.3850</td>
<td></td>
</tr>
<tr>
<td>APOC2 ENST00000252490</td>
<td>0.7010</td>
<td></td>
</tr>
<tr>
<td>APOE ENST00000252486</td>
<td>0.8855</td>
<td></td>
</tr>
<tr>
<td>ATP2B1 ENST00000261173 or NM_001682</td>
<td>0.2835</td>
<td>0.3450</td>
</tr>
<tr>
<td>CETP ENST00000200676 or NM_000078</td>
<td>0.9837</td>
<td>0.9450</td>
</tr>
<tr>
<td>CLMN ENST00000556454</td>
<td>0.8287</td>
<td></td>
</tr>
<tr>
<td>COQ2 ENST00000311469</td>
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<tr>
<td>CPT2 ENST00000371486</td>
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<td></td>
</tr>
<tr>
<td>DMPK ENST00000600757 or NM_001081563</td>
<td>0.0917</td>
<td>0.0134</td>
</tr>
<tr>
<td>DNAJC5B ENST00000519330</td>
<td>0.4474</td>
<td></td>
</tr>
<tr>
<td>GATM ENST00000396659</td>
<td>0.6792</td>
<td></td>
</tr>
<tr>
<td>HMGCR ENST00000287936 or NM_000859</td>
<td>0.9842</td>
<td>0.8845</td>
</tr>
<tr>
<td>HNRNPA1 ENST00000547276 or NM_002136</td>
<td>0.9989</td>
<td>0.6184</td>
</tr>
<tr>
<td>HTR3B ENST00000260191</td>
<td>0.9430</td>
<td></td>
</tr>
<tr>
<td>HTR7 ENST00000277874 or NM_000872</td>
<td>0.3918</td>
<td>0.0473</td>
</tr>
<tr>
<td>KIF6 ENST00000373213 or NM_001289020</td>
<td>0.1453</td>
<td>0.0015</td>
</tr>
<tr>
<td>LDLR ENST00000252444 or NM_001195799</td>
<td>0.6544</td>
<td>0.0973</td>
</tr>
<tr>
<td>LPA ENST00000316300</td>
<td>0.8790</td>
<td></td>
</tr>
<tr>
<td>LPIN1 ENST00000256720 or NM_145693</td>
<td>0.0121</td>
<td>0.0186</td>
</tr>
<tr>
<td>MYLIP ENST00000349606</td>
<td>0.6356</td>
<td></td>
</tr>
<tr>
<td>NOS3 ENST00000297494 or NM_000603</td>
<td>0.9306</td>
<td>0.7174</td>
</tr>
<tr>
<td>PCSK9 ENST00000302118</td>
<td>0.2274</td>
<td></td>
</tr>
<tr>
<td>PYGM ENST00000164139 or NM_001164716</td>
<td>0.6174</td>
<td>0.9177</td>
</tr>
<tr>
<td>RYR1 ENST00000600337 or NM_001042723</td>
<td>0.3779</td>
<td>0.0305</td>
</tr>
</tbody>
</table>
Figure 2. Total Expression (Ensembl-based) of ABCB1 by Age Group. Pairwise comparison p-values presented above box-plots.

Table 6. Mean Expression by Age Group for Genes of Significance, Excluding Prenatal Samples. Zero imputed for negative values in lower limit of CI.

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>Mean (95% CI), TPM</th>
<th>Kruskal Wallis p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1 Total Expression</td>
<td>1</td>
<td>5.583 (3.86, 7.31)</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.475 (7.34, 11.61)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.545 (8.35, 12.74)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12.650 (8.51, 16.79)</td>
<td></td>
</tr>
<tr>
<td>ABCB1 ENST00000265724</td>
<td>1</td>
<td>3.551 (2.31, 4.79)</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.696 (4.93, 8.47)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.847 (5.88, 9.81)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.899 (5.86, 11.94)</td>
<td></td>
</tr>
<tr>
<td>KIF6 NM_001289020</td>
<td>1</td>
<td>1.415 (0.50, 2.33)</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.008 (0, 0.02)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.025 (0, 0.05)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.036 (0, 0.12)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. Total Expression (UCSC-based) of ABCB1 by Age Group.

Figure 4. Expression (Ensembl-based) of ABCB1 Primary Transcript by Age Group. Pairwise comparison p-values presented above box-plots.
The UCSC dataset lists only one transcript for \textit{ABCB1}, so an additional primary transcript analysis was not performed. In the UCSC dataset analysis, \textit{KIF6} NM_001289020 showed significant association with age (p=0.0015) (Figure 5; Table 5). \textit{KIF6} expression was highest in Group 1 (<1 year old), which significantly differed from other groups (Table 6). Across all age groups, expression of \textit{KIF6} was relatively low (< 5 TPM).

\textbf{KIF6 Primary Transcript Expression by Age, UCSC}

![KIF6 Primary Transcript Expression by Age, UCSC](image)

Figure 5. Expression (UCSC-based) of \textit{KIF6} Primary Transcript by Age Group. Pairwise comparison p-values presented above box-plots.

\textbf{HMGCR and HNRNPA1 Alternative Transcript Expression}

There were no significant differences in the relative expression of alternative and canonical transcripts of \textit{HMGCR} or \textit{HNRNPA1} (HMGCR\textsubscript{Δ13}:HMGCR\textsubscript{ex13} or HNRNPA1\textsubscript{ex8}:HNRNPA1\textsubscript{Δ8}) associated with \textit{HMGCR} or \textit{HNRNPA1} genotypes in the general genetic model including the full set of liver tissue samples (Table 7). A trend toward an increase
in relative HMGCRΔ13 was seen with presence of ‘A’ alleles in HMGCR, but this relationship did not achieve significance (p=0.1015). The Caucasian subgroup general genetic model analysis did not show any significant trends (Table 8).

### Table 7. Alternative to Canonical Transcript Expression Ratio by Genotype, General Genetic Model.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean Ratio (95% CI)</th>
<th>Kruskal Wallis p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCRΔ13:HMGCRex13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3846662 G/G</td>
<td>0.294 (0.19, 0.45)</td>
<td>0.1015</td>
</tr>
<tr>
<td>rs3846662 G/A</td>
<td>0.459 (0.33, 0.64)</td>
<td></td>
</tr>
<tr>
<td>rs3846662 A/A</td>
<td>0.563 (0.33, 0.97)</td>
<td></td>
</tr>
<tr>
<td>HNRNPA1ex8:HNRNPA1Δ8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1920045 C/C</td>
<td>0.098 (0.07, 0.14)</td>
<td>0.5425</td>
</tr>
<tr>
<td>rs1920045 C/T</td>
<td>0.149 (0.09, 0.26)</td>
<td></td>
</tr>
<tr>
<td>rs1920045 T/T</td>
<td>0.112 (0.06, 0.20)</td>
<td></td>
</tr>
<tr>
<td>HMGCRΔ13:HMGCRex13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1920045 C/C</td>
<td>0.548 (0.42, 0.72)</td>
<td>0.1389</td>
</tr>
<tr>
<td>rs1920045 C/T</td>
<td>0.336 (0.23, 0.50)</td>
<td></td>
</tr>
<tr>
<td>rs1920045 T/T</td>
<td>0.373 (0.17, 0.82)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 8. Alternative to Canonical Transcript Expression Ratio by Genotype, General Genetic Model, Caucasian Subgroup.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean Ratio (95% CI)</th>
<th>Kruskal Wallis p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCRΔ13:HMGCRex13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3846662 G/G</td>
<td>0.173 (0.05, 0.60)</td>
<td>0.4187</td>
</tr>
<tr>
<td>rs3846662 G/A</td>
<td>0.273 (0.16, 0.47)</td>
<td></td>
</tr>
<tr>
<td>rs3846662 A/A</td>
<td>0.313 (0.09, 1.14)</td>
<td></td>
</tr>
<tr>
<td>HNRNPA1ex8:HNRNPA1Δ8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1920045 C/C</td>
<td>0.087 (0.02, 0.34)</td>
<td>0.9232</td>
</tr>
<tr>
<td>rs1920045 C/T</td>
<td>0.090 (0.05, 0.17)</td>
<td></td>
</tr>
<tr>
<td>rs1920045 T/T</td>
<td>0.093 (0.00, 0.31)</td>
<td></td>
</tr>
<tr>
<td>HMGCRΔ13:HMGCRex13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1920045 C/C</td>
<td>0.427 (0.18, 1.01)</td>
<td>0.1359</td>
</tr>
<tr>
<td>rs1920045 C/T</td>
<td>0.187 (0.11, 0.31)</td>
<td></td>
</tr>
<tr>
<td>rs1920045 T/T</td>
<td>0.213 (0.08, 0.59)</td>
<td></td>
</tr>
</tbody>
</table>

In the dominant genetic model including the full set of samples, expression of HMGCRΔ13:HMGCRex13 approached a significant association with HNRNPA1 genotype and HMGCR genotype (Table 9). Presence of at least one ‘T’ allele in HNRNPA1 approached an association with lower relative expression of HMGCRΔ13 (p=0.0470), while presence of at least one ‘A’ allele in HMGCR approached an association with higher relative expression of
HMGCRΔ13 (p=0.0465) (Figure 6,7). In the Caucasian subgroup analyses, no significant associations were found between genotypes and transcript expression ratios in the dominant model; however, the relationship between HNRNPA1 genotype and relative expression of HMGCRΔ13 was near significance (p=0.0500) (Table 10).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean Ratio (95% CI)</th>
<th>Wilcoxon p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCRΔ13:HMGCRex13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3846662 G/G</td>
<td>0.294 (0.19, 0.45)</td>
<td>0.0465</td>
</tr>
<tr>
<td>rs3846662 G/A, A/A</td>
<td>0.491 (0.38, 0.64)</td>
<td></td>
</tr>
<tr>
<td>HNRNPA1ex8:HNRNPA1Δ8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1920045 C/C</td>
<td>0.098 (0.07, 0.14)</td>
<td>0.3044</td>
</tr>
<tr>
<td>rs1920045 C/T, T/T</td>
<td>0.139 (0.09, 0.21)</td>
<td></td>
</tr>
<tr>
<td>HMGCRΔ13:HMGCRex13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1920045 C/C</td>
<td>0.548 (0.42, 0.72)</td>
<td>0.0470</td>
</tr>
<tr>
<td>rs1920045 C/T, T/T</td>
<td>0.345 (0.25, 0.48)</td>
<td></td>
</tr>
</tbody>
</table>

Table 9. Alternative to Canonical Transcript Expression Ratio by Genotype, Dominant Genetic Model.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean Ratio (95% CI)</th>
<th>Wilcoxon p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCRΔ13:HMGCRex13</td>
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<td></td>
</tr>
<tr>
<td>rs3846662 G/G</td>
<td>0.173 (0.05, 0.60)</td>
<td>0.1917</td>
</tr>
<tr>
<td>rs3846662 G/A, A/A</td>
<td>0.287 (0.19, 0.44)</td>
<td></td>
</tr>
<tr>
<td>HNRNPA1ex8:HNRNPA1Δ8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1920045 C/C</td>
<td>0.087 (0.02, 0.34)</td>
<td>0.7449</td>
</tr>
<tr>
<td>rs1920045 C/T, T/T</td>
<td>0.090 (0.05, 0.15)</td>
<td></td>
</tr>
<tr>
<td>HMGCRΔ13:HMGCRex13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1920045 C/C</td>
<td>0.427 (0.18, 1.01)</td>
<td>0.0500</td>
</tr>
<tr>
<td>rs1920045 C/T, T/T</td>
<td>0.192 (0.13, 0.28)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6. Ratio of Expression of HMGCRΔ13 to HMGCRex13 According to HMGCR Genotype, Dominant Model.

Figure 7. Ratio of Expression of HMGCRΔ13 to HMGCRex13 According to HNRNPA1 Genotype, Dominant Model.
CPR Clinical Analysis

Analysis of the associations between *HMGCR* or *HNRNPA1* genotypes and lipid values in the CPR showed that TC and LDL-C trended toward a significant relationship with the *HNRNPA1* genotype in a general genetic model (p=0.0325 and p=0.0177, respectively) (Table 11; Figure 8, 9). The heterozygous group had higher TC and LDL-C than either of the homozygous groups. No other significant associations were found in the analysis of the CPR as a whole.

<table>
<thead>
<tr>
<th>Table 11. Lipid Panel Values by Genotype, Total CPR.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene, SNP</strong></td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td><em>HMGCR</em>, rs3846662</td>
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<tr>
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<tr>
<td></td>
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<tr>
<td><em>HNRNPA1</em>, rs1920045</td>
</tr>
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</tbody>
</table>
Figure 8. Total Cholesterol Values by HNRNPA1 Genotype for All CPR Participants. Pairwise comparison p-value presented above box-plots.

Figure 9. LDL Cholesterol Values by HNRNPA1 Genotype for All CPR Participants. Pairwise comparison p-value presented above box-plots. There was a missing value for one participant.

After stratification by race, the African American subgroup showed a nearly significant association between HMGCR genotype with TC (p=0.0121), as well as with LDL-C (p=0.0309)
(Table 12; Figures 10, 11). Lipid levels trended toward an increase with the presence of the ‘A’ allele. Additionally, HNRNPA1 genotype showed a near significant association with TC in African Americans (p=0.0464) and LDL-C in Caucasians (p=0.0489) (Tables 12, 13; Figures 12, 13). In African Americans, TC was lower in the group homozygous for the ‘T’ allele compared to the heterozygous group. In Caucasians, LDL-C was highest in those with only one ‘T’ allele relative to those with either zero or two. After Bonferroni correction for multiple tests, the level of significance for CPR data was set at p<0.002. No relationships met this level of significance. There were no significant associations found between BMI category and either genotype before or after race stratification (Table 14).

Figure 10. Total Cholesterol Values by HMGCR Genotype for African American CPR Participants.
Figure 11. LDL Cholesterol Values by *HMGCR* Genotype for African American CPR Participants.
<table>
<thead>
<tr>
<th>Gene, SNP</th>
<th>Lipid</th>
<th>Genotype</th>
<th>Mean (95% CI), mg/dl</th>
<th>Wilcoxon p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>HMGCR</em>, rs3846662</td>
<td>TC</td>
<td>G/G</td>
<td>223 (204, 245)</td>
<td>0.0121</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/A</td>
<td>283 (226, 354)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>G/G</td>
<td>156 (138, 176)</td>
<td>0.0309</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>210 (145, 304)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>G/G</td>
<td>44 (39, 50)</td>
<td>0.3196</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>52 (40, 69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>G/G</td>
<td>99 (83, 119)</td>
<td>0.4541</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>79 (32, 192)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>HNRNPA1</em>, rs1920045</td>
<td>TC</td>
<td>C/C</td>
<td>N/A</td>
<td>0.0464</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>266 (222, 319)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>224 (202, 247)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>C/C</td>
<td>N/A</td>
<td>0.1112</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>193 (145, 258)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>156 (137, 178)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>C/C</td>
<td>N/A</td>
<td>0.7491</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>48 (41, 57)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>45 (38, 52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>C/C</td>
<td>N/A</td>
<td>0.2474</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>118 (84, 166)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>88 (70, 110)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 12. Total Cholesterol Values by *HNRNPA1* Genotype for African American CPR Participants.

Figure 13. LDL Cholesterol Values by *HNRNPA1* Genotype for Caucasian CPR Participants. Pairwise comparison p-value presented above box-plots.
### Table 13. Lipid Panel Values by Genotype, Caucasian Subgroup.

<table>
<thead>
<tr>
<th>Gene, SNP</th>
<th>Lipid</th>
<th>Genotype</th>
<th>Mean (95% CI), mg/dl</th>
<th>Kruskal Wallis p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HMGCR, rs3846662</strong></td>
<td>TC</td>
<td>G/G</td>
<td>241 (226, 256)</td>
<td>0.7182</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/A</td>
<td>235 (225, 245)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>233 (222, 243)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LDL-C</td>
<td>G/G</td>
<td>165 (153, 179)</td>
<td>0.3451</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/A</td>
<td>156 (148, 165)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>151 (142, 161)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HDL-C</td>
<td>G/G</td>
<td>45 (41, 49)</td>
<td>0.4428</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/A</td>
<td>45 (43, 48)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>47 (43, 51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>G/G</td>
<td>137 (117, 160)</td>
<td>0.7097</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/A</td>
<td>137 (120, 156)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>145 (121, 172)</td>
<td></td>
</tr>
<tr>
<td><strong>HNRNPA1, rs1920045</strong></td>
<td>TC</td>
<td>C/C</td>
<td>234 (223, 244)</td>
<td>0.5431</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/T</td>
<td>240 (230, 250)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>229 (213, 245)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LDL-C</td>
<td>C/C</td>
<td>155 (146, 164)</td>
<td>0.0489</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/T</td>
<td>163 (155, 172)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>146 (132, 161)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HDL-C</td>
<td>C/C</td>
<td>47 (43, 51)</td>
<td>0.517</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/T</td>
<td>45 (43, 47)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>46 (41, 51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>C/C</td>
<td>140 (122, 160)</td>
<td>0.5859</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/T</td>
<td>134 (118, 152)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>151 (117, 196)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 14. BMI Category by Genotype.

<table>
<thead>
<tr>
<th>Gene, SNP</th>
<th>Chi-square or Fisher's Exact P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All</strong></td>
<td></td>
</tr>
<tr>
<td><strong>HMGCR, rs3846662</strong></td>
<td>0.8702</td>
</tr>
<tr>
<td><strong>HNRNPA1, rs1920045</strong></td>
<td>0.1823</td>
</tr>
<tr>
<td><strong>Caucasians</strong></td>
<td></td>
</tr>
<tr>
<td><strong>HMGCR, rs3846662</strong></td>
<td>0.6824</td>
</tr>
<tr>
<td><strong>HNRNPA1, rs1920045</strong></td>
<td>0.2987</td>
</tr>
<tr>
<td><strong>African Americans</strong></td>
<td></td>
</tr>
<tr>
<td><strong>HMGCR, rs3846662</strong></td>
<td>*Fisher's exact test was performed due to counts &lt;5 in some cells</td>
</tr>
<tr>
<td><strong>HNRNPA1, rs1920045</strong></td>
<td>1.000</td>
</tr>
</tbody>
</table>

*Fisher’s exact test was performed due to counts <5 in some cells
DISCUSSION

It is widely accepted that gene expression changes during childhood growth and development. The study presented here set out to describe pediatric hepatic ontogeny for mRNA expression from 30 genes in pathways associated with cholesterol synthesis and statin response or toxicity, and explore the *in vivo* and *in vitro* presentation of genetic variation within two of these genes, *HMGCR* and *HNRNPA1*, during childhood.

When prenatal samples were included in the analyses, many genes showed a significant change in expression between age groups. This was expected due to the different cellular milieu of the prenatal liver compared to the post-natal liver. The function of the liver changes from hematopoietic (maximal activity around week 15) to gluconeogenic around birth. Each of the genes that showed significant expression changes in the ontogeny analysis with prenatal samples showed, as anticipated from the literature, that the prenatal age group was the source of the significant difference from at least one other group (Tables B1, B2). Additionally, the significant gene expression changes found using the Ensembl-based data were similar to those found with the UCSC-based data with a few exceptions that trended toward significance in one dataset and achieved significance in the other: *APOC2, CETP, COQ2*, and *LPIN1*. These slight differences with similar trends suggest that changes in expression are occurring, but they may not be significant at the mRNA level. Overall, however, the results of the prenatal sample analysis support the biological model that hepatocyte structure and function are dynamic during gestation and immediately following. Since transcriptomic analysis is only a marker for clinically impactful biological changes, further analysis in this area should focus on gene network and proteomic evaluation of the genes identified in this study.
When prenatal samples were excluded to examine differences only between postnatal groups, few individual genes showed significant hepatic expression changes; however, \textit{ABCB1}, which codes for a major drug efflux transporter, MDR1, did show an increase in mRNA expression with age in both the Ensembl-based total and primary transcript expression analyses (Table 4,6; Figures 2, 5). MDR1 is thought to transport some statins, as well as multiple non-statin drugs, such as methotrexate, out of the liver.\textsuperscript{16,31} A firmer understanding of the trajectory of \textit{ABCB1} expression may contribute to more effective pharmacotherapy for pediatrics. For instance, lower levels of \textit{ABCB1} expression may indicate a time of high hepatic accumulation of statins, and suggest the need for a lower dose. The UCSC database includes only one possible transcript for \textit{ABCB1}, while the Ensembl dataset includes 10. Since \textit{ABCB1} in the UCSC dataset showed a trend toward a significant increase in expression with age that did not achieve significance, further confirmation of the trend identified with the Ensembl dataset should be performed with a larger number of samples, focusing on the transcripts covered. The ontogenetic pattern found in this study supports the trend described by Mooij, \textit{et al.} and further differentiates between the expression levels at particular developmental stages.\textsuperscript{19} A proteomic study of hepatic MDR1 expression and activity could elucidate the functional implications of this mRNA expression change. Furthermore, although few individual genes showed significant expression changes with age, the sum of individually small changes may be acting in a network to affect the pathways associated with cholesterol metabolism and statin therapeutic response and toxicity. For this reason, gene network analysis should be performed with the RNA-seq dataset as the next step in examination of this dataset.

The associations between \textit{HMGCR} (rs3846662) or \textit{HNRNPA1} (rs1920045) genotype and the relative expression of alternative and canonical transcripts of \textit{HMGCR} and \textit{HNRNPA1} were
not found to be statistically significant in the pediatric liver samples. Burkhardt, *et al.* described how HMGCRΔ13 relative expression was increased in cells carrying an *HMGCR* ‘A’ allele in LCLs. Our results showed a trend of increased relative HMGCRΔ13 expression in pediatric liver samples that did not achieve statistical significance in either the general genetic or dominant *HMGCR* model. Yu, *et al.* described that the ‘T’ allele upstream of *HNRNPA1* is associated with altered constitutive expression of HNRNPA1ex8 in LCLs (Yu, *et al.* Supplemental Figure S6-A). Neither the general genetic model nor the dominant model supported an expression association between *HNRNPA1* genotype and relative expression of HNRNPA1ex8 in pediatric livers (*Tables 7, 9*). It was hypothesized that the ‘T’ allele should be expected to be associated with a decrease in constitutive relative HMGCRΔ13. No significant expression differences were detected between *HNRNPA1* genotype groups in the general genetic model, suggesting that, in pediatric hepatocytes, the associations are not strong. The dominant *HNRNPA1* genetic model, however, did show a trend toward a significant expression change, with decreased HMGCRΔ13 expression in the presence of one or two ‘T’ alleles, in line with the hypothesized effect. This result suggests that the hypothesized relationship between rs1920045 and *HMGCR* expression may be present in the pediatric population, but should be confirmed in a larger set of samples. The allele frequencies for both genotypes differ by race, which led to the decision to stratify the results by this factor. The dbSNP published frequencies of the *HMGCR* ‘G’ alleles in Caucasians and African Americans (population AoD) are 0.40 and 0.86, respectively. The dbSNP published frequencies of *HNRNPA1* ‘C’ alleles in Caucasians and African Americans (population AoD) are 0.63 and 0.29, respectively. In the Caucasian subgroup analyses, no significant associations between genotype and expression were found in either model. Analysis of the African American subgroup was not possible due to limited sample size. There are some limitations to our
genotype-associated expression analysis. The small sample size may have caused the race stratification analysis to be underpowered, thus masking significant trends. Additionally, the samples in this study spanned multiple developmental ages, however, small samples in each age/genotype group precluded stratification analysis. Preliminary investigation into the relative expression of HMGCRΔ13 across developmental ages trended toward, but did not reach significance, showing the need for further studies on the relationship between age and this expression (data not shown). An expansion of the sample size to include better representation of racial groups and developmental ages should be pursued in order to further evaluate the relationship between expression and genotype in children.

Evaluation of the relationships between HMGCR (rs3846662) and HNRNPA1 (rs1920045) genotypes and in vivo lipid levels revealed interesting associations. Based on adult studies and cell models, the HMGCR ‘A’ and HNRNPA1 ‘C’ alleles were anticipated to be associated with lower baseline total and LDL cholesterol values. In the CPR sample evaluated as a whole, no association was seen between baseline cholesterol values and HMGCR genotype. Near significant associations between HNRNPA1 genotype and TC or LDL-C were found. In both cases, the heterozygous group had higher TC and LDL-C values than either of the homozygous groups. Since the group showing different expression was heterozygous, this finding was inconclusive. Once the cohort was stratified by race, some of the associations between lipid panel values and genotype changed. The Caucasian subgroup analysis did not reveal any significant relationships between genotypes and age, although the relationship between LDL-C and HNRNPA1 genotype approached significance, with the same pattern of highest LDL-C values in the heterozygous group. In the African American subgroup, the relationships between HMGCR genotype and TC or LDL-C trended toward significance and
average levels increased with the presence of one ‘A’ allele. This was opposite of the expected relationship. Additionally, a trend toward a significant decrease in TC with the presence of the \textit{HNRNPA1} ‘T’ allele was noted. After the Bonferroni correction for multiple tests, no relationships met the criteria for significance, yet this is considered to be an overly-conservative adjustment for significance. The data showed preliminary evidence for interesting trends between \textit{HMGCR} or \textit{HNRNPA1} genotypes and lipid levels in children and revealed that race may be confounding the genotype-phenotype relationship. This information indicated that further studies should focus on ensuring adequate representation of racial groups when characterizing the clinical implications of these SNPs in pediatrics. An additional limitation in the evaluation of \textit{in vivo} lipid levels was that the study group overall had higher lipid levels than average, and there was no comparison to a group of children with “acceptable” lipid levels. Further investigations into this, and into trends in lipid levels over time, may better elucidate the relationship between lipid levels and these genotypes. One additional limitation is in the race stratification. Using self-reported race may not be as accurate as identifying race by genotyping, and including individuals reporting Hispanic ethnicity within their respective racial group, without stratification, may mask some relationships that could be present.

In conclusion, this study confirmed the presence of dynamic gene expression in the liver during childhood development; however the number of genes with expression changes after birth was less than anticipated. For this study, and many others, mRNA was used as a surrogate marker to investigate downstream changes, such as protein expression. Since mRNA is not translated at a universal rate and can be differentially degraded, the lack of mRNA expression changes in the pediatric hepatocytes may not actually indicate constant expression of transporter proteins or enzymes. Future studies should focus on protein activity changes throughout
development. In particular, the implications of \textit{ABCB1} mRNA expression increasing with age should be evaluated on a protein level, as the MDR1 transporter is involved with multiple medications, and this knowledge could contribute to pharmacokinetic models.

Furthermore, the information in this study suggests that the impact of genetic variation in pediatrics may not mimic that of adult relationships. Genotype-phenotype relationships that are significant in adult populations may not be significant in children, or the relationships may be altered. Our study showed that the expression changes associated with two SNPs in LCLs were not significant in pediatric hepatocytes, but certain relationships trended toward significance. \textit{In vivo}, the impact of these SNPs appeared to be confounded by race. Relationships approaching significance appeared after stratification, including two with phenotypes opposite of what had been hypothesized.

The challenges of establishing adequate sample sizes for full genomic studies in the pediatric setting highlights the importance of maximizing that data from each study. The indication that race and age may confound phenotypic presentation in individuals from this study points to the need to ensure adequate representation of different races and developmental stages in future pediatric pharmacogenomic studies.

When possible, data on established genotype-phenotype relationships from adult studies should be utilized to inform therapeutic interventions in children; however, only studies conducted in pediatric populations will accurately capture variability attributable to maturity, which is not evident from adult studies.
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APPENDIX A

List of Gene Abbreviations

ABCB1 – ATP-Binding Cassette, Sub-Family B, Member 1
AGTR1 – Angiotensin II Receptor, Type 1
AMPD1 – Adenosine Monophosphate Deaminase 1
APOC1 – Apolipoprotein C-I
APOC2 – Apolipoprotein C-II
APOE – Apolipoprotein E
ATP2B1 – ATPase, Ca++ Transporting, Plasma Membrane 1
CETP – Cholesterol Ester Transfer Protein, plasma
CLMN – Calmin
CPT2 – Carnitine Palmitoyltransferase 2
COQ2 – Coenzyme Q2 4-hydroxybenzoate Polyprenyltransferase
CYP7A1 – Cytochrome P450, Family 7, Subfamily A, Polypeptide 1
DMPK – Dystrophia Myotonica-protein Kinase
DNAJC5B – DnaJ(Hsp40) homolog, Subfamily C, Member 5 Beta
GATM – Glycine Aminotransferase
HMGCR – 3-Hydroxy-3-methylglutaryl CoA Reductase
HNRNPA1 – Heterogeneous Nuclear Ribonucleoprotein A1
HTR3B – 5-Hydroxytryptamine Receptor 3B, Ionotropic
HTR7 – 5-Hydroxytryptamine Receptor 7, Adenylate Cyclase-coupled
KIF6 – Kinesin Family Member 6
LDLR – Low Density Lipoprotein Receptor
LPA – Lipoprotein, Lp(a)

LPIN1 – Lipin 1

MDR1* – Multidrug Resistance Protein 1

MYLIP – Myosin Regulatory Light Chain Interacting Protein

NOS3 – Nitric Oxide Synthase 3

PCSK9 – Proprotein Convertase Subtilisin/Kexin Type 9

PYGM – Phosphorylase, Glycogen, Muscle

RyR1 – Ryanodine Receptor 1

SLC10A1 – Solute Carrier Family 10, Member 1

SLCO1B1 – Solute Carrier Organic Anion Transporter Family, Member 1B1

*Protein, not gene
## APPENDIX B

### Table B1. Pairwise Comparison Results for Total Gene Expression with Prenatal Samples by Age Group Analysis.

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