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PII: S0168-8278(15)00599-1
DOI: http://dx.doi.org/10.1016/j.jhep.2015.08.027
Reference: JHEPAT 5813

To appear in: Journal of Hepatology

Received Date: 5 March 2015
Revised Date: 21 August 2015
Accepted Date: 24 August 2015

Please cite this article as: Younossi, Z.M., Stepanova, M., Estep, M., Negro, F., Clark, P.J., Hunt, S., Song, Q., Paulson, M., Stamm, L.M., Brainard, D.M., Mani Subramanian, G., McHutchison, J.G., Patel, K., Dysregulation of Distal Cholesterol Biosynthesis in Association with Relapse and Advanced Disease in CHC Genotype 2 and 3 Treated with Sofosbuvir and Ribavirin, Journal of Hepatology (2015), doi: http://dx.doi.org/10.1016/j.jhep.2015.08.027

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Dysregulation of Distal Cholesterol Biosynthesis in Association with Relapse and Advanced Disease in CHC Genotype 2 and 3 Treated with Sofosbuvir and Ribavirin

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Word Count: 4,220  Abstract: 240  Tables and Figures: 3+3

Funding source: This study was funded by Gilead Sciences

Disclosures: ZMY, FN, PC and KP have received research funding from Gilead. QS, MP, LS, DB, MS2 and JGM are employees of Gilead. MS1, ME and SH have no conflict of interest

Keywords: HCV, sofosbuvir, ribavirin, lipid metabolism
ABSTRACT

BACKGROUND AND AIMS: Hepatitis C virus (HCV) modulates host lipid metabolism for its replication and lifecycle. Our aims were to assess changes in the serum lipid and distal (post-squalene) cholesterol biosynthesis metabolite profile of HCV genotypes (GT) 2 and 3 patients treated with sofosbuvir+ribavirin. METHODS: Serum samples [baseline, treatment week 12, 4 weeks post-treatment] were analyzed for apolipoproteins B and E (apoB/E), total cholesterol, HDL, LDL, and 11 post-squalene sterol metabolites using a GC/MS platform. RESULTS: We selected 127 patients (GT2 N=50, GT3 N=77), 50% cirrhotic, and 42% who experienced a virological relapse. At baseline, GT3 patients had lower level of serum lipids, apoB/E, 7-dehydrocholesterol, desmosterol, lathosterol, compared to GT2 (p<0.006). Baseline lathosterol was lower in relapsers with cirrhosis compared to cirrhotics with SVR (p=0.003). From baseline to treatment week 12, serum lipids, apoB/E, and key sterol pathway metabolites (7-dehydrocholesterol, desmosterol, lathosterol, lanosterol) increased in GT3. In contrast, in GT2 patients, apoB/E and dihydrolanosterol decreased with viral suppression (p<0.025). At follow-up week 4, cirrhotic SVR patients showed substantially greater increases in apoB and total sterols compared to cirrhotic relapsers regardless of HCV genotype. After adjustment for genotype and gender, baseline lathosterol was independently associated with virologic response (p=0.04). CONCLUSION: HCV genotype 3 is associated with reduced circulation of lipids involved in the distal cholesterol biosynthesis pathway, resulting in relative hypocholesterolemia. HCV suppression during sofosbuvir+ribavirin restores distal sterol metabolites indicating viral interference with de novo lipogenesis or selective retention by hepatocytes.
INTRODUCTION

Hepatitis C virus (HCV) is a positive-strand RNA hepatotropic virus that modulates host lipid metabolism as a part of its natural lifecycle from virion entry, polyprotein processing and replication, to assembly and release [1-5]. Chronic hepatitis C (CHC) infection is associated with dyslipidemia and metabolic features that include insulin resistance, reduced serum total cholesterol, low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B levels [6-7]. These metabolic disturbances can lead to hepatic steatosis. Both viral- and host-related factors can lead to the disease progression and poor virologic response to interferon (IFN)-based therapy [8-11].

There are interesting lipid and metabolic phenotype differences between HCV genotypes. Hepatic steatosis, reduced VLDL secretion [12-13] and hypocholesterolemia appear to predominate in HCV genotype (GT) 3 infection. These metabolic sequelae of HCV GT3 infection are mostly reversible following viral eradication [14], and may be due to direct viral inhibition of microsomal triglyceride transfer protein (MTTP) [15-16].

HCV replication is dependent upon geranylgeranyl, an isoprenoid product of the mevalonate pathway (or cholesterol biosynthesis) [17]. Post-translational modification of geranylgeranyl facilitates the membrane association between a key protein, F-box and leucine-rich repeat protein 2 (FBL2) with HCV NS5A [18]. However, the role of FBL2 in viral replication has not been fully established across all HCV genotypes. Thus, although HCV seems to increase cholesterol synthesis, the hypocholesterolemia commonly seen in HCV patients may be secondary to an impaired secretion of lipoproteins from hepatocytes, leading to steatosis [14].

Desmosterol is a major cholesterol synthesis intermediate (Figure 1) [19], and a prior study demonstrated downregulation of HCV replication in the JFH-1 cell culture model through the
inhibition of desmosterol metabolism [20]. A small pilot study demonstrated HCV GT3-specific perturbation of distal cholesterol biosynthesis metabolites but the preservation of proximal metabolites such as lanosterol [21]. However, the pathogenic mechanisms and reasons for this genotype-specific inhibition of cholesterol biosynthesis pathway to provide a survival advantage to the virus are poorly understood.

During the significant shift in the treatment paradigm to oral direct-acting antiviral (DAA) therapy in CHC infection, HCV GT3-infected patients, particularly those with treatment-experience and/or cirrhosis, have emerged as a more difficult to treat population requiring longer duration therapy [22-23]. The role of cholesterol biosynthesis in relation to lower virologic response rates to current DAA therapy in HCV GT3 patients has not been previously defined. Our hypothesis was that GT3-selective disturbance in distal cholesterol pathway metabolites observed in blood is (1) further exacerbated in HCV GT3-infected patients with cirrhosis and, (2) is associated with higher relapse rates following IFN-free DAA therapy. Our aims were to assess genotype-specific changes in serum lipids and sterol metabolites in HCV GT2 and GT3 patients based on the disease severity and/or the response to treatment with sofosbuvir and ribavirin.

**METHODS**

**Study population**

The selected study cohort included adult CHC GT2 and GT3 patients with available sera who received oral sofosbuvir (SOF) and ribavirin (RBV) IFN-free therapy for 12 or 16 weeks in three multicenter, phase 3 clinical registration trials: POSITRON, FISSION and FUSION [22,24]. The study cohort was selected retrospectively to include both SVR and relapse patients and to reduce variability in treatment type, duration, and related outcomes amongst HCV GT2 and GT3 patients who were enrolled in these trials. The FISSION study cohort included treatment-naïve HCV GT2
and GT3 patients [24], POSITRON included patients who were ineligible to receive or intolerant to a prior interferon (IFN)-containing therapy [22], and the FUSION cohort included patients who did not have a response to a prior IFN-based treatment [22]. Patients on HMGCoA reductase inhibitors or other lipid lowering therapy during the study were excluded (Supplementary Figure 1).

Samples (non-fasting) were collected at baseline, treatment week 12 (TW12), and post-treatment follow-up (FU) week 4. Serum was isolated from whole blood following clotting and centrifugation and stored immediately at -70°C until thawed for analyses. HCV RNA levels were measured by the COBAS Ampliprep/COBAS TaqMan 2.0 assay (Roche Molecular Systems, Pleasanton, CA) with a lower limit of quantification of 25 IU/mL. Fibrosis assessment was by liver biopsy, or standard thresholds for FibroScan (EchoSens, Paris, France) [25] and/or FibroTest (BioPredictive, Paris, France) [26].

These ancillary studies were approved by the institutional review boards or independent ethics committees at each participating clinical site and were conducted in compliance with Good Clinical Practice guidelines and local regulatory requirements. This sub-study was granted an exempt status by Inova Institutional Review Board.

**Lipid panel and sterol metabolites**

Apolipoprotein E (apoE), total cholesterol and HDL were all analyzed by Covance Central Laboratory Services (CCLS) using Roche Modular Analyzer and the appropriate immunoturbidimetric or enzymatic/colorimetric assay. CCLS measured apolipoprotein B (apoB) concentrations by immunonephelometry using the BNII Nephelometer (Siemens Healthcare Diagnostics, Tarrytown, NY).
Eleven serum metabolites in the post-squalene cholesterol synthetic pathway were quantified using ion-ratio gas chromatography-mass spectrometry on an Agilent 6390N/5973 Gas Chromatography/Mass Spectrometer (GC-MS) system as previously described [27]. These included key sterol metabolites lanosterol, lathosterol, desmosterol, 7-dehydrocholesterol (7-DHC) and cholesterol that were assessed from blood samples in the initial analysis. Additional analysis included other intermediate metabolites (8-dehydrocholesterol, 8(9)-cholestenol, dihydrolanosterol, 4α-methyl-5α-cholesta-7-en-3β-ol and, 4α-methyl-5α-cholesta-8-en-3β-ol). Two phytosterols (campesterol and sitosterol) were measured as markers of cholesterol intestinal absorption, and in combination with the biosynthesis markers above, provided an overall assessment of cholesterol metabolism.

**Statistical analysis**

Demographic parameters, lipids and sterol metabolites at different time points, as well as changes in those from patients' own baseline, were compared between those with HCV GT2 and GT3 using chi-square tests for homogeneity and Mann-Whitney non-parametric tests. Changes from baseline in all studied parameters were compared to zero using the Wilcoxon sign rank test, and statistical significance would indicate a significant change from patients' own baseline levels. The pairwise comparisons were also conducted stratified by cirrhosis and SVR. False discovery rate (FDR) was calculated using the Benjamini-Hochberg procedure for the studied 15 sterols and metabolites, three time points, and 12 rounds of comparisons based on the presence of SVR and/or cirrhosis and/or GT.

Independent predictors of lipids and sterol metabolites at each time point were assessed using multiple linear regression. Bidirectional stepwise selection with the significance level of 0.2 for entry and of 0.05 for stay was used. Potential predictors included age, gender, race/ethnicity,
cirrhosis, pre-treatment history of diabetes, hyperlipidemia and hypertension, HCV genotype, and SVR (post-treatment only). All statistical analyses were run in SAS 9.3 (SAS Institute, Cary, NC) and statistical significance was assessed at a p-value of 0.05.

Pathway analysis
Pathway analysis utilized MetaCore™ software (Thomson Reuters Corp., New York, NY) and KEGG pathways. Initially, KEGG entry IDs were manually obtained for each relevant analyte and cross-referenced with PubChem IDs and uploaded to MetaCore. ApoB and apoE were uploaded separately as expression type data. To assess distinctions at baseline between HCV GT2 and GT3 patients, significantly differentially circulating analytes were further evaluated using various functional ontology enrichment tools, including Pathway and Pathway Folder (Process) analysis, Disease (by biomarker) analysis, and Metabolic analysis. This process was repeated for those analytes that displayed significantly differential dynamics from baseline to post-treatment between the GT 3 patients that achieved SVR and relapers. When appropriate, MetaCore results were also manually compared to KEGG pathways for union.

RESULTS
Patient Demographics
The study cohort included 127 CHC patients (GT2: N=50, GT3: N=77) who received SOF and RBV regimen (FUSION N=38, POSITRON N=87 and FISSION N=2). Subjects were predominantly Caucasian (89%) and male (61%), with age 54.4 ± 8.4 years, BMI 28.3 ± 5.1. Overall, 58.3% (N=74) achieved SVR and 41.7% relapsed during post-treatment follow-up. The prevalence of cirrhosis was 50% in our study cohort (GT2: N=27, GT3: N=37), and 37/64 of cirrhotics (57.8%) achieved SVR.
In our patient population, HCV GT3 patients were generally younger (52.7 ± 8.3 vs. 56.9 ± 7.9 years, p=0.002), had a higher proportion with elevated ALT (75% vs. 46%; p<0.001; ALT 112.1 ± 70.2 U/L vs. 82.7 ± 62.0 U/L, p=0.005), and lower SVR rates (48.1% vs. 74.0%, p=0.004) compared to GT2 (Table 1). By design, other demographic characteristics were similar between the genotypes (Table 1).

**Baseline Differences in Lipids and Sterol Metabolites by Genotype and Cirrhosis**

At baseline, serum apoB, apoE, total cholesterol, LDL, total sterols and key post-squalene metabolites (lanosterol, lathosterol, 7-DHC, and desmosterol) were all lower in GT3 compared to GT2 (Table 2). However, among the 64 patients with cirrhosis, only apoE and total cholesterol were significantly lower in patients with GT3 compared to GT2 (Table 2). There were no other differences in the post-squalene sterol metabolites between cirrhotic patients with HCV GT2 or GT3, although our ability to detect significant differences for this subpopulation may have been limited by the smaller cohort size (Table 2).

To address whether viral genotype-specific decrease of specific circulating lipids is related to advanced disease, we compared lipids and sterol metabolites between cirrhotic and non-cirrhotic patients. Patients with GT3 cirrhosis (N=37) had lower HDL (40.8 ± 14.4 vs. 49.5 ± 15.7 mg/dL; p=0.024) and the pre-lathosterol intermediate (4α-methyl-5α-cholesta-7-en-3β-ol) (0.11 ± 0.19 vs. 0.16 ± 0.15 μg/mL; p=0.016), but higher level of an intestinal absorption marker sitosterol (2.5 ± 1.2 vs. 1.8 ± 0.8 μg/mL; p=0.020), compared to GT3 non-cirrhotic patients (N=40). The only apparent difference in lipids and sterol metabolites for GT2 patients was a lower 4α-methyl-5α-cholesta-7-en-3β-ol in GT2 cirrhotics (N=27) compared to GT2 non-cirrhotics (N=23) (0.09 ± 0.16 vs 0.20 ± 0.20 μg/mL; p=0.028).
In a multiple linear regression model, factors independently associated with baseline lathosterol (in µg/mL) were Caucasian race ($\beta = -0.46 \pm 0.21$, $p=0.030$) and GT3 ($\beta = -0.33 \pm 0.13$, $p=0.015$). HCV GT3 was also a predictor of baseline apoB (mg/dL) ($\beta = -13.06 \pm 3.95$, $p=0.0012$), apoE ($\beta = -0.79 \pm 0.25$, $p=0.0018$), total cholesterol (mg/dL) ($\beta = -31.7 \pm 6.2$, $p<0.0001$), LDL (mg/dL) ($\beta = -22.0 \pm 5.3$, $p=0.0001$), and desmosterol (µg/mL) ($\beta = -0.17 \pm 0.05$, $p=0.0019$). Furthermore, cirrhosis was associated with $4\alpha$-methyl-$5\alpha$-cholesta-7-en-3β-ol (µg/mL) ($\beta = -0.08 \pm 0.03$, $p=0.012$), campesterol (µg/mL) ($\beta = 0.85 \pm 0.38$, $p=0.0256$), and sitosterol (µg/mL) ($\beta = 0.54 \pm 0.21$, $p=0.0134$).

**Baseline Differences in Lipids and Sterol Metabolites Based on Treatment Response**

An overall comparison between the 74 (58%) SVR patients and 53 (42%) relapsers in our study cohort indicated that lathosterol (1.4 ± 0.8 vs. 1.1 ± 0.5 µg/mL, $p=0.023$) and a pre-lathosterol intermediate $4\alpha$-methyl-$5\alpha$-cholesta-8-en-3β-ol (0.16 ± 0.57 vs. 0.03 ± 0.15 µg/mL, $p=0.020$) along with total cholesterol (164 ± 34 vs. 146 ± 39 mg/dL, $p=0.009$) were all higher at baseline in patients who achieved SVR compared to those who did not. The same pre-lathosterol intermediate was higher in GT3 patients with SVR (N=37) (0.12 ± 0.26 vs 0.03 ± 0.17 µg/mL, $p=0.031$) compared to GT3 relapers (N=40). Differences in sterol metabolites were not apparent for HCV GT2 patients; only HDL (52.5 ± 20.1 vs. 39.6 ± 19.5 mg/dL, $p=0.028$) and lathosterol (1.6 ± 0.9 vs. 1.2 ± 0.6 µg/mL, $p=0.042$) were marginally elevated at baseline in GT2 patients who achieved SVR compared (N = 37) to relapers (N = 13).

For the cirrhotic cohort (N=64), LDL (93.6 ± 28.6 vs. 76.6 ± 29.5 mg/dL, $p=0.033$), total cholesterol (163.5 ± 30.2 vs. 140.9 ± 34.7 mg/dL, $p=0.0079$) and lathosterol (1.42 ± 0.72 vs. 0.94 ± 0.48 µg/mL; $p=0.0031$) were higher at baseline in SVR patients (N=37) compared to relapers (N=27). However, there were no significant differences in baseline sterol metabolites between SVR and relaper GT3 cirrhosis patients.
Genotype-Associated Changes in Lipid and Sterol Metabolites Following Treatment

At TW12, HCV GT3 patients had significant increases from baseline in apoB, total cholesterol, HDL and LDL, key distal sterol metabolites, along with a decrease in apoE (all p<0.05: Table 3, Supplementary Table 2). There were further increases through FU week 4 in apoB, LDL, HDL, total cholesterol, and distal sterol metabolites, except dihydrolanosterol and lanosterol (all p<0.03: Figure 2). In contrast, HCV GT2 patients had significant decreases in apoB and -E from baseline at TW12 (all p<0.013: Table 3, Supplementary Table 2). Additionally, all lipids and metabolites, except for apoE, increased to baseline levels by FU week 4 (all p<0.03). Amongst the post-squalene sterol metabolites, there was a decrease in dihydrolanosterol for HCV GT2 patients at TW12, and increases in lathosterol and two pre-lathosterol intermediates at FU week 4 (p<0.022: Supplementary Table 2).

We next assessed for genotype-specific changes in lipids and sterol metabolites at TW12 and FU week 4 in relation to SVR. However, there was no significant difference in change from baseline in lipid or sterol metabolites at TW12 between SVR and relapers for either genotype. In particular, for GT3 subjects, similar decreases in apoE, and similar increases in total cholesterol, HDL, LDL, 7-dehydrosterol, campesterol, desmosterol, and lathosterol were observed (all p<0.05 for comparison to patients’ own baseline levels, all p>0.05 for comparison between SVR and relapser GT3 patients) (Supplementary Table 1). By FU week 4, GT3 patients who achieved SVR had greater increases in apoB, desmosterol, lathosterol, and cholesterol than GT3 relapers (all p<0.022: Supplementary Table 1). There were no significant differences between GT2 SVR and GT2 relapers at treatment week 12 or FU week 4 (all p>0.05).

Change in Sterol Metabolites and Lipids Following Treatment in Cirrhosis Patients
Overall, there was no significant difference in change from baseline in sterol metabolites or lipids to TW12 between cirrhosis patients with SVR (N=37/64) or relapse (N=27/64) (all p>0.05). However, at FU week 4, increases in apoB (+9.1 ± 17.8 vs. -2.2 ± 13.0 mg/dL, p=0.002), total cholesterol (+24.6 ± 28.9 vs. +5.9 ± 27.0 mg/dL, p=0.007), and LDL (+19.5 ± 28.1 vs. +6.0 ± 23.7 mg/dL, p=0.040) were greater in cirrhosis patients with SVR compared to relapsers.

At TW12, compared to GT3 cirrhotic patients (N=37), GT3 patients without cirrhosis (N=40) had a greater increase from baseline in sitosterol (+0.43 ± 0.69 vs. -0.06 +/- 0.86 µg/mL, p=0.029). At FU week 4, increases in both plant sterols sitosterol (+0.50 ± 0.72 vs. -0.11 ± 1.00 µg/mL, p=0.0044) and campesterol (+0.95 ± 1.32 vs. -0.13 ± 1.96 µg/mL, p=0.0094) were again greater in non-cirrhotic GT3 patients. Other changes from baseline at TW12 or FU4 were similar between GT3 cirrhotic and non-cirrhotic patients (all p>0.05).

There were no difference in changes in sterol metabolites and lipids following treatment between GT2 patients with (N=27) and without (N=23) cirrhosis (all p>0.05).

In GT3 patients with cirrhosis (N=37), no difference between SVR (N=18/37) and relapse (N=19/37) patients were noted at TW12. However, by FU4, GT3 cirrhotic patients with SVR experienced greater increases in apoB, total cholesterol, LDL, and desmosterol compared to GT3 cirrhotic relapsers (all p<0.05) (Supplementary Table 1).

Finally, after a total of twelve rounds of GT+SVR+cirrhosis-based comparisons of all the sterols and metabolites at three time points (a total of 540 comparisons), we received 75 potentially significant findings with p<0.05; the resulting FDR is estimated to be 35%. In the same series of analysis, the FDR=25% would require considering p<0.025 only.
Pathway analysis of lipids and sterol metabolites related to SVR and presence of cirrhosis

Pathway analysis of the baseline lipids and sterol metabolites between GT2 and GT3 patients implies significant differential regulation of several key pathways involved in cholesterol biosynthesis and transport. In particular, GT3 patients who achieved SVR showed significantly greater increases between pre- and post-treatment levels for several sterol metabolites compared to GT3 relapers (Figure 3). As expected in this study of distal pathway metabolites, pathway analysis reveals that the differentially expressed analytes are downstream of the C4 demethylation complex and/or the enzyme 3β-hydroxysterol Δ24-reductase (DHCR24) that catalyzes both early reduction of lanosterol, and late stage reduction of desmosterol as the final step in the Bloch cholesterol synthesis pathway (Figure 1).

Lipids and Sterol Metabolites Associated with Treatment Response

In multivariate logistic regression analysis of SVR, after adjustment for gender (female: OR (95%CI) = 3.57 (1.51-8.42), p=0.0037) and GT2 (OR = 3.64 (1.53-8.67), p=0.0035), there was an independent association between baseline lathosterol level and SVR (OR = 2.08 (1.03-4.18) per µg/mL, p=0.041). There were no other associations between SVR and lipid or sterol metabolites at baseline or changes in those at TW12 (all p>0.05). However, an increase in desmosterol by FU week 4 was independently associated with SVR in GT3 patients (OR=21.3 (2.5-180.9) per each additional µg/mL, p=0.0050).

DISCUSSION

This study provides new insights into the selective dysregulation of post-squalene sterol and lipid metabolism in HCV GT3 patients with (1) advanced disease, and (2) in relation to virologic
response to sofosbuvir and ribavirin therapy. Prior to treatment, serum post-squalene sterol metabolites were all lower in GT3 patients. We also observed novel specific baseline differences in pre-lathosterol intermediates for both GT2 cirrhotics and GT3 cirrhotics, and among GT3 patients who achieved SVR compared to relapsers. Higher baseline lathosterol was independently associated with SVR. As expected, serum lipid and sterol metabolites increased following therapy in GT3, along with further increases in patients who did not relapse following treatment.

Hepatitis C modulates host lipid metabolism for its lifecycle [1,28]. Circulating low-density HCV lipoviroparticles are associated with apoB-containing lipoproteins and demonstrate higher infectivity in cell culture [29-30]. For reasons that remain unclear, HCV GT3 is known to be associated with lower LDL, hypobetalipoproteinemia, and increased steatosis due to viral-mediated inhibition of microsomal triglyceride transfer protein [16] and other mechanisms [7]. Another lipoprotein, apoE, is an important lipid transport protein and constituent of VLDL, binds to LDL-R, and is involved in mediating HCV cell entry and viral assembly through an interaction with HCV NS5A [31-33]. In our study, patients with GT3 and cirrhosis had lower apoE and cholesterol at baseline compared to patients with GT2 with cirrhosis. Although some apoE isoforms have been associated with fibrosis in HCV GT1, we did not measure these isoforms in our cohort of HCV GT3 patients [6, 8, 10, 11, 13, 14, 34-37].

An interesting issue is the potential interaction between HCV and the cholesterol biosynthesis pathway. This pathway is complex and involves multiple enzymatic reactions. The third step, catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoS-R) is often regarded as the rate-limiting step, and there are important associations with HCV infection (Figure 1) [17, 38]. Certainly, in vitro inhibition of HMGCoS-R by statins leads to reduced HCV RNA [39-40]. This may occur through inhibition of geranylgeranylation of FBL2 and reduced interaction with the HCV
non-structural protein 5A [18]. However, the antiviral efficacy of statin monotherapy in HCV patients has proved to be insignificant [41-42]. Regulation of cholesterol synthesis also occurs at other stages in the pathway [43]. The post-squalene pathway distal to HMCoA-R is important in regulation of cholesterol synthesis, and the predominant location of these enzymes in the endoplasmic reticulum also allows for possible interaction with HCV. The first sterol intermediate is lanosterol formed in a two-step process from the isoprenoid squalene. The first step involves a key enzyme, squalene monoxygenase, which has also been proposed as a second rate limiting enzyme in cholesterol synthesis [43]. The lanosterol-to-cholesterol pathway is also complex, involving 19 enzymatic reactions. Lanosterol is either diverted into the Bloch pathway, leading to cholesterol formation through desmosterol, or the Kandutsch-Russell pathway through 7-dehydrocholesterol [43]. Our study indicated that the distal post-squalene sterol metabolites lathosterol, desmosterol and 7-DHC were lower in GT3 patients compared to GT2. A prior small study in 33 CHC patients (GT2=13, GT3=20) treated with IFN-based therapy indicated no baseline genotype differences in distal sterol metabolites, except for 7-DHC [21]. In contrast, our study included a larger cohort with both fibrosis assessment and sterol metabolite measurement at end-of-treatment, to allow for clinically relevant subgroup analyses in relation to genotype, relapse, and advanced disease. In addition, our study also demonstrated on-treatment increases in desmosterol and lanosterol for GT3 patients, and a novel independent association between baseline lathosterol and SVR. Another study in 5 CHC patients used stable isotope mass spectrometry to assess plasma fatty acid composition, and demonstrated reduced cholesterol synthesis despite increased hepatic lipogenesis, compared to healthy controls [44]. However, sterol metabolites were not assessed. Our study indicated marginal genotype differences in levels of lanosterol which is an important feedback regulator of HMGCoA-R. Oxidative stress has been associated with HCV, impairs sterol regulatory element-binding protein (SREBP) transcriptional regulation of sterol synthesis, and maintains lanosterol levels [45-47]. This should downregulate HMGCoA-R activity, but perhaps HCV hypocholesterolemia allows balanced
HMGCoA-R activity, thus allowing ongoing synthesis of pre-squalene isoprenoids that are important for HCV replication. We observed an increase in lanosterol levels in HCV GT3 but not GT2 patients at week 12, further supporting genotype-specific regulation. However, any potential role of HCV in regulation of lanosterol levels has yet to be defined.

Targeting downstream, post-lanosterol, pathways reduces HCV replication in cell culture, and appears synergistic with other prenylation inhibitors. Desmosterol is a key sterol intermediate and is particularly interesting in relation to HCV replication. A study using the HCV Japanese fulminant hepatitis strain 1 (JFH-1) cell culture model indicated that HCV replication is dependent upon desmosterol production through 7-dehydrocholesterol reductase (DHCR7) in the Bloch synthesis pathway [20]. Other studies indicate that 24-dehydrocholesterol reductase (DHCR24) that catalyzes conversion of desmosterol to cholesterol, is an important mediator of lipid raft formation [19], overexpressed in HCV infection, and is associated with viral replication [48-49]. However, we did not observe any specific differences in measured post-lanosterol intermediates, and all key distal sterol metabolites, including desmosterol, were reduced in HCV GT3 patients at baseline. The increase in these distal sterols and cholesterol levels following HCV RNA clearance by week 12 in HCV GT3 patients suggests viral-specific inhibition of post-squalene sterol biosynthesis, or virally mediated selective retention of cholesterol and distal sterols by hepatocytes. The reasons for this selective genotype associated perturbation of sterols is not clear, and to further characterize the role of SREBP, microRNAs, Liver X receptors, and LDL-R mediated transport in HCV intracellular sterol homeostasis would require significant in-depth transcriptional and post-translational cellular profiling of the distal pathway. Recent studies identified the association of HCV and forkhead box transcription factors, and their interactions with HCV core protein [50]; however, no HCV genotype specific differences were identified.
Interestingly, we observed an increase in both sitosterol and campesterol in GT3 non-cirrhotics following treatment. Although these dietary phytosterols contribute to an insignificant proportion of the total cholesterol pool, they are surrogate markers for intestinal cholesterol absorption [51], and have not been previously evaluated in HCV infection. Sterol absorption is regulated by intestinal transporters, and since we observed an increase in phytosterols campesterol and sitosterol in GT3 patients at TW12, this suggests possible viral inhibition of intestinal efflux and biliary transporters [52]. Our observation also reflects change in cholesterol biosynthesis with therapy in GT3 patients, that likely influences cholesterol absorption to maintain relative lipid homeostasis.

There were minor decreases at baseline in a pre-lathosterol intermediate (4α-methyl-5α-cholesta-7-en-3β-ol) in patients with both GT2 and GT3 cirrhosis, but other pre- and post-lathosterol intermediates were preserved. However, another pre-lathosterol intermediate (4α-methyl-5α-cholesta-8-en-3β-ol) was lower at baseline in GT3 patients that relapsed following therapy. Lathosterol is an established marker of whole-body cholesterol synthesis [53]. Baseline lathosterol levels were independently associated with SVR and also lower in cirrhosis patients who relapsed compared to those who achieved SVR, suggesting lowered de novo sterol biosynthesis in these patients. Due to the relatively small cohort, we were not able to demonstrate genotype-specific differences in baseline lathosterol levels and virologic response among cirrhotic patients. Other host variables such as IFNL3 gene variants have been associated with higher levels of LDL-C and ApoB for HCV GT1 but not GT3 [54]. However, there was no association between IFNL3 rs12979860 genotype with relapse in our cohort, and was not further evaluated in relation to sterol intermediates.

In summary, this study demonstrates reduced de novo lipogenesis in HCV GT3 may be due to disturbance in the distal cholesterol biosynthesis pathway, or a selective derangement of hepatocyte lipid secretion resulting in relative hypocholesterolemia. Lathosterol and pre-lathosterol
intermediate metabolites are lower in GT3 patients, and lathosterol levels lower in cirrhotics who relapse following 12 or 16 weeks of sofosbuvir and ribavirin therapy. Both the pathogenic mechanisms underlying genotype-specific modulation of host lipid biosynthesis for replication advantage and the need for longer duration therapy for GT3 require further assessment. This may provide clue as to why genotype 2 behaves differently than genotype 3. However, given the rapid advent of next generation pangenotypic combination DAA therapies, genotype-dependent lipid metabolism differences may be less important. Nevertheless, the impact of different all-oral regimens for treatment of HCV genotype 3 may provide additional interesting insights.

REFERENCES


**Table 1.** Patients’ demographics.

<table>
<thead>
<tr>
<th></th>
<th>HCV genotype 3</th>
<th>HCV genotype 2</th>
<th>p-value</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>77</td>
<td>50</td>
<td></td>
<td>127</td>
</tr>
<tr>
<td>Age, years</td>
<td>52.7 ± 8.3</td>
<td>56.9 ± 7.9</td>
<td>0.0019</td>
<td>54.4 ± 8.4</td>
</tr>
<tr>
<td>BMI</td>
<td>27.9 ± 4.9</td>
<td>29.0 ± 5.4</td>
<td>0.25</td>
<td>28.3 ± 5.1</td>
</tr>
<tr>
<td>Male</td>
<td>47 (61.0%)</td>
<td>31 (62.0%)</td>
<td>0.91</td>
<td>78 (61.4%)</td>
</tr>
<tr>
<td>White</td>
<td>70 (90.9%)</td>
<td>43 (86.0%)</td>
<td>0.39</td>
<td>113 (89.0%)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>37 (48.1%)</td>
<td>27 (54.0%)</td>
<td>0.51</td>
<td>64 (50.4%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>12 (15.6%)</td>
<td>3 (6.0%)</td>
<td>0.10</td>
<td>15 (11.8%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>17 (22.1%)</td>
<td>16 (32.0%)</td>
<td>0.21</td>
<td>33 (26.0%)</td>
</tr>
<tr>
<td>Hyperlipidemia *)</td>
<td>3 (3.9%)</td>
<td>5 (10.0%)</td>
<td>0.17</td>
<td>8 (6.3%)</td>
</tr>
<tr>
<td>ALT (≥ ULN)</td>
<td>58 (75.3%)</td>
<td>23 (46.0%)</td>
<td>0.0008</td>
<td>81 (63.8%)</td>
</tr>
<tr>
<td>SVR24</td>
<td>37 (48.1%)</td>
<td>37 (74.0%)</td>
<td>0.0038</td>
<td>74 (58.3%)</td>
</tr>
</tbody>
</table>

*) None taking HMGC0A reductase inhibitors or other lipid lowering therapy
Table 2. Baseline genotype differences in lipids and sterol metabolites (mean ± standard deviation).

<table>
<thead>
<tr>
<th></th>
<th>GT3 All (n=79)</th>
<th>GT2 All (n=54)</th>
<th>p-value GT3 vs. GT2 (all)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GT3 Cirrhosis (n=37)</th>
<th>GT2 Cirrhosis (n=27)</th>
<th>p-value GT3 vs. GT2 (cirrhosis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein B, mg/dL</td>
<td>67.7 ± 20.4</td>
<td>81.6 ± 23.2</td>
<td>0.0009</td>
<td>70.5 ± 21.4</td>
<td>80.7 ± 25.3</td>
<td>0.14</td>
</tr>
<tr>
<td>Apolipoprotein E, mg/dL</td>
<td>4.27 ± 1.18</td>
<td>5.12 ± 1.60</td>
<td>0.0052</td>
<td>4.30 ± 1.08</td>
<td>5.17 ± 1.25</td>
<td>0.0051</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>144.0 ± 32.1</td>
<td>175.8 ± 35.9</td>
<td>&lt;0.0001</td>
<td>144.1 ± 28.9</td>
<td>167.48 ±</td>
<td>0.0053</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>45.1 ± 15.6</td>
<td>49.3 ± 20.5</td>
<td>0.40</td>
<td>40.8 ± 14.4</td>
<td>46.4 ± 18.0</td>
<td>0.23</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>77.9 ± 27.9</td>
<td>99.9 ± 30.4</td>
<td>0.0002</td>
<td>79.6 ± 26.8</td>
<td>96.7 ± 32.0</td>
<td>0.06</td>
</tr>
<tr>
<td>Total Sterols, µg/ml</td>
<td>1593 ± 354</td>
<td>1926 ± 400</td>
<td>&lt;0.0001</td>
<td>1640 ± 332</td>
<td>1840 ± 416</td>
<td>0.047</td>
</tr>
<tr>
<td>7-Dehydrocholesterol, µg/ml</td>
<td>0.292 ± 0.476</td>
<td>0.493 ± 0.683</td>
<td>0.0001</td>
<td>0.364 ± 0.679</td>
<td>0.355 ±</td>
<td>0.11</td>
</tr>
<tr>
<td>8(9)Choles-tene-3-ol, µg/ml</td>
<td>0.215 ± 0.204</td>
<td>0.263 ± 0.214</td>
<td>0.07</td>
<td>0.262 ± 0.270</td>
<td>0.163 ±</td>
<td>0.49</td>
</tr>
<tr>
<td>4amethyl 5acholesta -7-en 3b-ol, µg/ml</td>
<td>0.137 ± 0.169</td>
<td>0.141 ± 0.185</td>
<td>0.87</td>
<td>0.106 ± 0.186</td>
<td>0.158 ±</td>
<td>0.66</td>
</tr>
<tr>
<td>4amethyl 5acholesta -8-en 3b-ol, µg/ml</td>
<td>0.072 ± 0.220</td>
<td>0.158 ± 0.677</td>
<td>0.72</td>
<td>0.094 ± 0.271</td>
<td>0.190 ±</td>
<td>0.20</td>
</tr>
<tr>
<td>Campesterol, µg/ml</td>
<td>3.84 ± 2.19</td>
<td>4.22 ± 2.06</td>
<td>0.22</td>
<td>4.37 ± 2.36</td>
<td>4.49 ± 2.32</td>
<td>0.78</td>
</tr>
<tr>
<td>Desmosterol, µg/ml</td>
<td>0.736 ± 0.269</td>
<td>0.905 ± 0.321</td>
<td>0.0016</td>
<td>0.764 ± 0.284</td>
<td>0.331 ±</td>
<td>0.22</td>
</tr>
<tr>
<td>Dihydrolanosterol, µg/ml</td>
<td>0.008 ± 0.009</td>
<td>0.011 ± 0.019</td>
<td>0.13</td>
<td>0.009 ± 0.012</td>
<td>0.006 ±</td>
<td>0.58</td>
</tr>
<tr>
<td>Lanosterol, µg/ml</td>
<td>0.067 ± 0.044</td>
<td>0.082 ± 0.057</td>
<td>0.049</td>
<td>0.069 ± 0.045</td>
<td>0.029 ±</td>
<td>0.50</td>
</tr>
<tr>
<td>Lathosterol, µg/ml</td>
<td>1.19 ± 0.62</td>
<td>1.53 ± 0.87</td>
<td>0.0024</td>
<td>1.14 ± 0.73</td>
<td>1.34 ± 0.58</td>
<td>0.07</td>
</tr>
<tr>
<td>Sitosterol, µg/ml</td>
<td>2.11 ± 1.06</td>
<td>2.29 ± 1.45</td>
<td>0.63</td>
<td>2.46 ± 1.20</td>
<td>2.44 ± 1.33</td>
<td>0.62</td>
</tr>
</tbody>
</table>
Table 3. Genotype-associated change in sterol metabolites and lipids following treatment.

<table>
<thead>
<tr>
<th></th>
<th>GT3 (N=77)</th>
<th>GT2 (N=50)</th>
<th>GT3 vs. GT2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ baseline</td>
<td>Δ baseline</td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein B, mg/dL</td>
<td>5.5 ± 17.0</td>
<td>-5.7 ± 13.4</td>
<td>0.0083</td>
</tr>
<tr>
<td></td>
<td>0.041</td>
<td>0.0083</td>
<td>0.0018</td>
</tr>
<tr>
<td>Apolipoprotein E, mg/dL</td>
<td>-0.79 ± 0.99</td>
<td>-1.31 ± 0.97</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.014</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>20.7 ± 32.7</td>
<td>-7.0 ± 23.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>5.8 ± 12.2</td>
<td>2.0 ± 9.0</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>0.047</td>
<td>0.04</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>14.8 ± 25.1</td>
<td>-4.8 ± 20.1</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Δ Follow-up wk 4c

<table>
<thead>
<tr>
<th></th>
<th>Δ week12</th>
<th>Δ week12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein B, mg/dL</td>
<td>12.3 ± 18.7</td>
<td>1.8 ± 16.0</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>Apolipoprotein E, mg/dL</td>
<td>-0.12 ± 0.99</td>
<td>-0.38 ± 1.08</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>0.013</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>29.9 ± 37.0</td>
<td>6.1 ± 24.9</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>5.5 ± 11.4</td>
<td>2.0 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>0.035</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>23.9 ± 30.6</td>
<td>4.8 ± 23.7</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

a- Change from patient’s own baseline to treatment week 12
b- p-value for the significance of the change from baseline
c- Change from week 12 to post-treatment follow-up week 4
NS: not significant (p≥0.05)
Figure 1. Overview of the cholesterol synthesis pathway. Note: Dashed lines indicate multi-step reaction. Steroid intermediates in boxes; those with red outlines were significantly lower at baseline in GT3 patients compared to GT2; those with red text increased more significantly in GT3 patients who achieved SVR compared to relapsers (p<0.05).

*Phytosterols are produced in plants distal to a squalene branch point. Circulating phytosterols were measured in this study to indicate dietary intake of steroids and metabolites.

Figure 2: Changes from baseline throughout post-treatment (FU4) in sterol metabolites in patients with HCV GT3.

Figure 3: Post-treatment (FU4) changes from baseline in sterol metabolites in patients with HCV GT3 with and without SVR (p<0.05 only).
Figure 2: Changes from baseline throughout post-treatment (FU4) in sterol metabolites in patients with HCV GT3. Error bars represent ± standard error of the mean as a percent.
Figure 3: Post-treatment (FU4) changes from baseline in sterol metabolites in patients with HCV GT3 with and without SVR (p<0.05 only). Error bars represent standard error of the mean.