# Achieving sustained virologic response after interferon-free hepatitis C virus treatment correlates with hepatic interferon gene expression changes independent of cirrhosis

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SUMMARY. Chronic hepatitis C virus (HCV) infection can now be treated with oral directly acting antiviral agents, either with or without ribavirin (RBV). Virologic relapse after treatment can occur, and in some studies was more common in cirrhotic subjects. We previously observed changes in hepatic immunity during interferon (IFN)-free therapy that correlated with favourable outcome in subjects with early liver disease. Here, we compared changes in endogenous IFN pathways during IFN-free, RBV-free therapy between cirrhotic and noncirrhotic subjects. mRNA and microRNA (miRNA) expression analyses were performed on paired pre- and post-treatment liver biopsies from genotype-1 HCV subjects treated with sofosbuvir/ledipasvir (SOF/LDV) for 12 weeks ($n = 4$, 3 cirrhotics) or SOF/LDV combined with GS-9669 or GS-9451 for 6 weeks ($n = 6$, 0 cirrhotics). Nine of ten subjects achieved a sustained virologic response (SVR), while one noncirrhotic subject relapsed. Hepatic IFN-stimulated gene expression decreased with treatment in the liver of all subjects, with no observable impact of cirrhosis. Hepatic gene expression of type III IFNs (*IFNL1*, *IFNL3*, *IFNL4-ΔG*) similarly decreased with treatment, while *IFNA2* expression, undetectable in all subjects pretreatment, was detected post-treatment in three subjects who achieved a SVR. Only the subject who relapsed had detectable *IFNL4-ΔG* expression in post-treatment liver. Other IFNs had no change in gene expression (*IFNG*, *IFNB1*, *IFNA5*) or could not be detected. Although expression of multiple hepatic miRNAs changed with treatment, many miRNAs previously implicated in HCV replication and IFN signalling had unchanged expression. In conclusion, favourable treatment outcome during IFN-free HCV therapy is associated with changes in the host IFN response regardless of cirrhosis.

*Keywords:* cirrhosis, endogenous interferons, hepatitis C virus, interferon-free therapy, treatment relapse.

Rates of sustained virologic response (SVR) after treatment of chronic hepatitis C virus (HCV) infection have improved

markedly with use of interferon (IFN)-free regimens composed of directly acting antiviral agents (DAAs). The most frequent cause of treatment failure is virologic relapse within 12 weeks of completing treatment. Subjects with advanced liver disease and prior nonresponse to IFN-based therapy experienced higher rates of treatment relapse in some studies (reviewed in [1]), although mechanisms are unclear. Increased risk for relapse in cirrhotics was most pronounced when first generation protease inhibitors were combined with IFN-based therapy [2–5], but has also been observed with sofosbuvir (SOF)- and simeprevir-based regimens [6–9]. Choice of DAA composition and treatment duration for approved HCV genotype-1 (GT1) regimens factor cirrhosis and history of nonresponse to IFN-based therapy, aiming to maximizing SVR rates for populations based on results from comparative clinical trials [10,11].

We previously showed that intrahepatic and peripheral immunity change rapidly after starting DAA therapy, with endogenous IFN activity in blood and liver declining within days of treatment in parallel with HCV suppression [12]. Whole tissue transcriptional analysis suggested hepatic type III IFNs played a prominent role in hepatic inflammation during chronic infection and that induction of a type I IFN response during HCV suppression was associated with SVR [12,13]. In peripheral blood, restoration of polyfunctional anti-HCV-specific CD8 T-cell function occurred during DAA treatment with greater frequency in subjects who achieved SVR than relapsed [14]. Cirrhosis is considered a state of relative immunosuppression, with defects in innate and adaptive immunity [1], and impaired expression of type I IFN receptor (*IFNAR1*) has been observed in HCV-infected cirrhotic subjects [15]. Whether cirrhosis impairs host immunity in a way that impacts treatment outcome for HCV infection with DAA therapy is unknown.

Our previous study examining endogenous IFN changes in paired pre- and post-treatment liver biopsies of subjects treated with SOF and ribavirin (SOF/RBV) in the National Institutes of Health (NIH) SPARE trial did not include biopsies from cirrhotic subjects [12]. Thus, although all 4 subjects with cirrhosis in SPARE relapsed [6], the impact of cirrhosis on endogenous IFNs could not be addressed [12]. We were also unable to assess the influence of RBV on hepatic immunity, as all subjects in SPARE received either low (600 mg daily) or weight-based (1000–1200 mg daily) RBV. RBV retains an important role for multiple DAA regimens in trials and clinical practice, appears to reduce the odds of relapse in some studies [7,11,16], and has been shown to modulate intrahepatic immunity and increase IFN sensitivity [17–20]. In the current study, we hypothesized that cirrhosis and the use of RBV-free DAA regimens might impact changes in innate immunity during IFN-free HCV treatment. To address this hypothesis, we used paired pre- and post-treatment liver biopsies from cirrhotic and noncirrhotic subjects treated with combination IFN-free, RBV-free DAA therapy in the NIH SYNERGY trial and examined hepatic mRNA and miRNA species associated with endogenous IFN signalling.

## MATERIALS AND METHODS

### Clinical trial and samples

Study subjects were enrolled in a phase 2, single-centre, prospective, community-based clinical trial (SYNERGY, NCT01805882) that evaluated all-oral DAA treatment at the National Institute of Allergy and Infectious Diseases (NIAID), NIH in Bethesda, MD. Sixty GT1, treatment-naïve, HCV-mono-infected subjects were treated with SOF and ledipasvir (LDV) for 12 weeks (Arm A, $n = 20$) or SOF/ LDV combined with the investigational NS3/4A inhibitor GS-9451 (80 mg) (Arm B, $n = 20$) or the allosteric NS5B inhibitor GS-9669 (250 mg twice daily) (Arm C, $n = 20$) for 6 weeks [21]. All subjects provided written or oral informed consent approved by the NIAID/NIH Institutional Review Board (IRB). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the NIAID IRB [21]. As previously reported, 58 of 60 subjects achieved SVR with treatment [21].

Paired pre- and post-treatment liver biopsies were evaluated from 10 subjects, 9 of whom achieved SVR. Pretreatment liver biopsies were obtained within 1.2 years of starting therapy and post-treatment biopsies were obtained within 5 days of completing treatment. The subject who relapsed had a post-treatment liver biopsy 1 day after completing treatment, while relapse was detected 2 weeks post-treatment at his subsequent follow-up appointment. Histopathologic assessment of liver biopsies was performed by a single nonblinded pathologist at the time of biopsy and was staged by the Knodell histological activity index and ISHAK scoring systems [22].

### mRNA and small RNA isolation

Core liver biopsies obtained transcutaneously with an 18-gauge needle were immediately placed in RNAlater (Qiagen, Valencia, CA, USA) and stored at $-80$ °C until shipment on dry ice to Rocky Mountain Labs, Genomics Unit, Research Technology Section. Half of each biopsy specimen was homogenized in 1 mL of TRIzol (ThermoFisher Scientific, Waltham, MA, USA) in a FastPrep Green lysing matrix vial (40 s, setting 6) using a FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA, USA), and was then combined with 200 μL of 1-bromo-3-chloropropane (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 4 °C at 16 000×g for 15 min. The RNA containing aqueous phase (520–600 μL) was collected and passed through a Qiashredder column (Qiagen) at 21 000×g for 2 min. RNA was extracted using the RNeasy 96 kit including an on-column DNase I digestion (Qiagen). RNA quality was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the Agilent RNA 6000 Pico kit. The average RNA Integrity Number value was 8.5, with a range of 7.5–9.2. Liver biopsy small RNA (0–200 nucleotides) were extracted according to the Qiagen miRNA protocol. Briefly, sample RLT/ethanol flow-through was combined with 180 μL RLT and 780 μL 100% ethanol, centrifuged through an RNeasy minicolumn, washed per protocol and then eluted with RNase-free water.

### Microarray analysis

DNA microarray targets were synthesized from 5 ng of total RNA according to the manufacturer's instructions

using the Ovation Pico WTA system version 2 RNA kit (Nugen Inc., San Carlos, CA, USA), including four *Bacillus subtilis* polyA-tailed mRNAs as technical controls to monitor cDNA synthesis and amplification during target preparation. Amplified single-stranded cDNAs (ss-cDNAs) were purified according to the QIAquick 96-well protocol (Qiagen) with a modified centrifugation step [23]. Sample quantity and purity were measured using the SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA, USA). ss-cDNAs were of high quality based on testing with the Agilent 2100 Bioanalyzer. The average size of ss-cDNA was above 340 bp, which is the recommended size by the kit manufacturer (Nugen Inc.), and all cDNA targets were similar in size.

Labelled target ss-cDNA pools were assayed on the Affymetrix GeneChip Human Gene 2.0 ST array according to the manufacturer's instructions. Quality analysis was performed according to the 'Quality Assessment of Exon and Gene Arrays' (Affymetrix revision 1.1, Santa Clara, CA, USA). All .cel files were normalized and uploaded into the GEO database (study GSE70779). Quality assessment indicated successful normalization but identified one outlier with excessive background signal, which along with its paired biopsy sample were excluded from subsequent mRNA analysis. Data were imported and analysed in Partek Genomics Suite software (version 6.6; Carlsbad, CA, USA). A paired *t*-test considering the 9-paired biopsies was multiple-test-corrected using the false discovery rate (FDR) step-up method [24]. This approach produced only five transcripts with an FDR <0.05 (*DDX60, STAT1, IRF9, LAP3, HLAC*), and only 3 of these had a fold change >2 (*DDX60, STAT1, IRF9*). Therefore, an input of the top 1% ranked probe sets (*n* = 536) at 1.25X was used for downstream analysis with Ingenuity Pathway Analysis (IPA, Qiagen), as previously described [12]. The molecule activator pathway of IPA was used for predicted cytokine activation state, with values over 2 considered significant based on IPA criteria.

## Quantitative reverse transcriptase–polymerase chain reaction

Expression analysis was performed with custom (*IFNL3* and *IFNL4*) or predesigned TaqMan assays using a 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA), as previously described [12,25]. Total RNA from liver was reverse-transcribed using random primers with the High Capacity cDNA Reverse Transcriptase Kit (Life Technologies). Twenty ng of RNA was used for *IFNA2*, *IFNL3* and *IFNL4* amplification reactions. Between 5 and 20 ng was used for other genes, with assays run as a single replicate due to limited sample availability. Gene expression was determined as a cycle at threshold (Ct) based on 40 PCR cycles. *GAPDH* Ct values, used as an endogenous control, were distributed between 22 and 25

without clear differences between samples. Delta Ct values are reported relative to *GAPDH* expression [12]. In cases where endogenous IFNs were undetectable in most samples, qualitative data are presented as 'detectable' *vs* 'undetectable'. Quantitative expression and statistical analysis is reported for IFNs with consistently detectable gene expression.

## Small RNA sequencing and miRNA analysis

Small RNAs isolated from the 20 samples were sequenced using the Illumina TruSeq Small RNA protocol according to the manufacturer's instructions. Libraries were size selected in the range of 140–340 base pairs, of which 122 bases were adaptor sequence. Libraries were run on a Hiseq 2500 as 50-base pair single-end reads. Sequencing generated a total of 749 881 628 reads with an average of 37 494 081 reads per sample.

Sequencing reads were trimmed of adaptor sequence, and reads <16 nucleotides were removed. Reads were filtered for low quality (FASTX-Toolkit), resulting in an average of 36 328 014 reads remaining in each sample. Reads were mapped to a database of rRNA, tRNA and repetitive sequences (RepBase19) using Bowtie2 (-L 16 – score-min L, 0, −0.15). Remaining reads (average 15 933 747 per sample) were mapped to build hg19 of the human genome using Bowtie2 (-L 16 – score-min L, 0, −0.15) with an average alignment rate of 88%. Reads mapping to mature miRBase (v20), SNORs (small nucleolar RNAs) (NCBI) and long intergenic noncoding RNAs (lincRNAs) (Broad Institute, Cambridge, MA, USA) were counted. Low expression miRNAs were filtered out, with criteria that at least five samples had two counts per million for an individual miRNA, and then normalized by the trimmed mean of *M*-values method using the EdgeR Bioconductor package. Paired *t*-test (unadjusted *P*-value) and FDR-corrected *P*-values (*q*-values) were computed using Partek Genomic Suite, with significant species identified with a *q*-value <0.05. Only miRNA results are reported here. No technical outliers were identified, and data from all 10 subjects were included in the analysis. The sequencing data were deposited at the National Center for Biotechnology Information Sequence Read Archive under accession number SRP061390.

## RESULTS

We analysed paired pre- and post-treatment liver biopsies from subjects with chronic HCV GT1 infection to explore the impact of cirrhosis on endogenous IFN signalling during IFN-free, RBV-free DAA treatment. Liver samples from subjects treated in a phase-2 clinical trial (SYNERGY) with 12 weeks of SOF/LDV or 6 weeks of SOF/LDV combined with GS-9669 or GS-9451 were analysed (subject characteristics shown in Table 1) [21]. Most subjects were male

(8/10), African American (8/10), had unfavourable *IFNL4-ΔG* genotype (8/10 with at least 1 *IFNL4-ΔG* allele), 3/10 had biopsy-proven cirrhosis (all enrolled in Arm A), and 9/10 achieved SVR. In the overall SYNERGY clinical trial, 58 of 60 subjects achieved SVR [21], and the only treatment relapser was included in the cohort of subjects with paired liver biopsies that was analysed here [21].

**Table 1** Subject demographics for paired liver microarrays

| Subject ID* | Age (yrs) | Gender | Race† | HCV genotype | *IFNL4* genotype‡ | Treatment arm§ | ISHAK fibrosis (pre/post)¶ | Cirrhotic | HAI inflammation (pre/post)¶ | Treatment outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 (1, 11) | 56 | M | W | 1a | ΔG/TT | A | 6/6 | Yes | 10/7 | SVR |
| 2 (2, 12) | 47 | M | AA | 1a | ΔG/TT | A | 6/6 | Yes | 7/5 | SVR |
| 3 (3,13) | 62 | M | AA | 1a | ΔG/TT | A | 6/6 | Yes | 7/2 | SVR |
| 4 (4, 14) | 59 | M | AA | 1b | TT/TT | A | 2/0 | No | 10/3 | SVR |
| 5 (5, 15) | 55 | F | AA | 1b | ΔG/TT | B | 4/4 | No | 8/5 | SVR |
| 6 (6, 16) | 61 | M | AA | 1a | ΔG/TT | B | 4/3 | No | 11/10 | Relapse |
| 7** | 56 | F | AA | 1a | ΔG/TT | C | 0/0 | No | 4/5 | SVR |
| 8 (8, 18) | 52 | M | W | 1a | TT/TT | C | 2/2 | No | 11/5 | SVR |
| 9 (9, 19) | 55 | M | AA | 1a | ΔG/ΔG | B | 2/1 | No | 8/9 | SVR |
| 10 (10, 20) | 52 | M | AA | 1a | ΔG/ΔG | C | 4/4 | No | 7/10 | SVR |

*Identification codes for subject microarray data available in the GEO database (study GSE70779). †W (White), AA (African American). ‡*IFNL4* genotype determined by the rs368234815 variant (TT/ΔG). §Treatment arm: A (SOF/LDV for 12 weeks), B (SOF/LDV + GS-9669 for 6 weeks), C (SOF/LDV + GS-9451 for 6 weeks). ¶ISHAK fibrosis (0–6) and histological activity index (HAI) (0–18) scores on pre- and post-treatment liver biopsies. **Sample 7 was excluded from microarray analysis, but included for miRNA analysis, as described in the methods section.
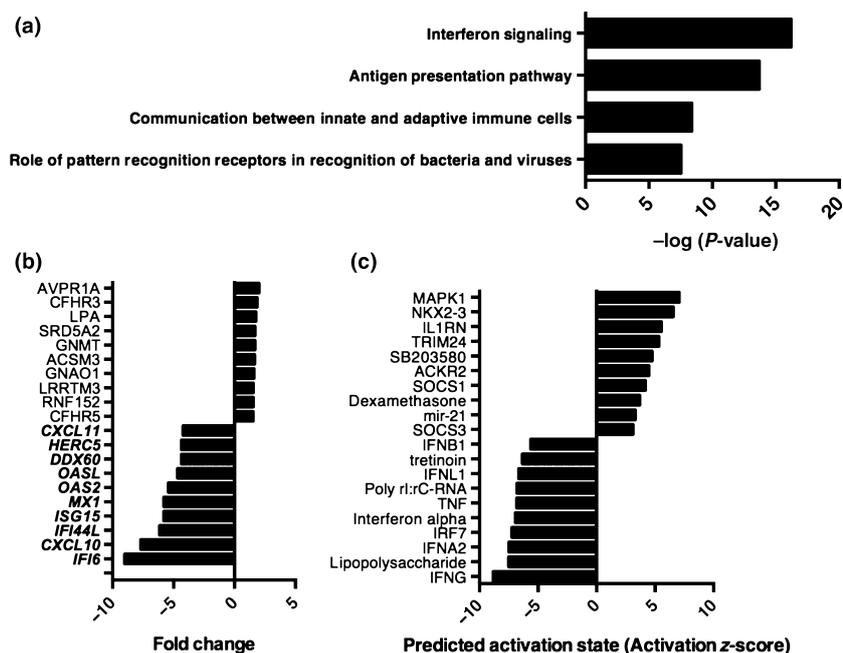


**Fig. 1** Endogenous interferon signalling declines with treatment. Paired liver biopsies from nine subjects (eight sustained virologic response, one relapse) were analysed by microarray gene expression analysis. The top 1% of genes identified by a rank-order approach were analysed using Ingenuity Pathway Analysis. (**a**) The top 4 canonical pathways as ranked by *P*-value. All had predicted reduced activity. (**b**) The top 10 significant upregulated and downregulated genes (IFN-stimulated genes are shown with bold/italicized font) based on fold change. (**c**) The top significant 10 upregulated and downregulated 'predicted activation state' based on z-score.

In histological assessment of paired liver biopsies, no subjects were found to have had a significant change in fibrosis over the course of treatment, while 7 of 10 subjects experienced a reduction in HAI inflammation (Table 1). Microarray analysis of mRNA from paired liver biopsies revealed downregulated expression of genes associated with IFN signalling, antigen presentation and pattern recognition of viruses as a result of treatment (Fig. 1a), consistent with previous results from noncirrhotic GT1 subjects treated with SOF/RBV [12]. IFN-stimulated genes (ISGs) represented the individual genes with the highest downregulation of expression (Fig. 1b). Type I (IFNA2, IFNB1), type II (IFNG) and type III (IFNL1) signalling pathways were all predicted to have reduced activation in hepatic tissue as a result of treatment (Fig. 1c).

To examine whether cirrhosis might impact the degree to which IFN signalling is downregulated, select ISGs were amplified by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) on individual samples. We found near uniform downregulation of ISG expression in all subjects, irrespective of cirrhosis, and no difference in pre- or post-treatment expression of ISGs between cirrhotics and noncirrhotics, or based on pretreatment viral load (Fig. 2). While the subject who relapsed had high expression of ISGs pretreatment, similar downregulation was observed compared to subjects achieving SVR (Fig. 2), consistent with the notion that global ISG expression is downregulated in nearly all subjects irrespective of treatment outcome. USP18, an ISG that negatively regulates type I IFN signalling, was also downregulated, consistent with the hypothesis that type I IFN sensitivity has the potential to be restored during DAA therapy [12].

Previous quantification of hepatic IFN gene expression from subjects who achieved SVR with SOF/RBV treatment revealed reduced levels of most type III IFNs over the course of treatment, while upregulation of IFNA2 was observed in some subjects who achieved SVR [12]. As RBV can modulate sensitivity to endogenous IFNs [19,26,27], we explored differential gene expression of endogenous IFNs in this RBV-free trial, and extended the analysis to include additional type I IFN genes. While IFNA2 gene expression was undetectable in all subjects pretreatment, IFNA2 was detected in 3 of 9 subjects post-treatment, all who achieved SVR and one of whom was cirrhotic (Fig. 3a). In contrast, gene expression of type III IFNs was detected by qRT–PCR in many subjects pretreatment, but was undetectable or had reduced expression in post-treatment samples (Fig. 3a and b). Pretreatment IFNLs were readily detected in most cirrhotic subjects, as 2 of 3 subjects with IFNL1 and 3 of 6 subjects with IFNL4-ΔG detected were cirrhotic. IFNA5 showed a trend towards transcriptional upregulation with treatment ($P = 0.09$), while no change was observed in IFNB1 or IFNG expression, and most other type I IFNs (IFNA1, IFNA4, IFNA6, IFNA8, IFNA16, IFNA17) and IFNL2 could not be detected either pre- or post-treatment (Fig. 3b and data not shown). Post-treatment IFNL4-ΔG was only detected in the subject who experienced treatment relapse. This finding parallels that observed in SOF/RBV treated subjects with paired liver biopsies, in which IFNL4-ΔG was detectable in post-treatment liver of the subject who relapsed, but in only 1 of 7 subjects who achieved SVR [12].

We next investigated differential expression of miRNAs associated with HCV suppression during DAA therapy, as over half of the genes implicated in innate immunity have
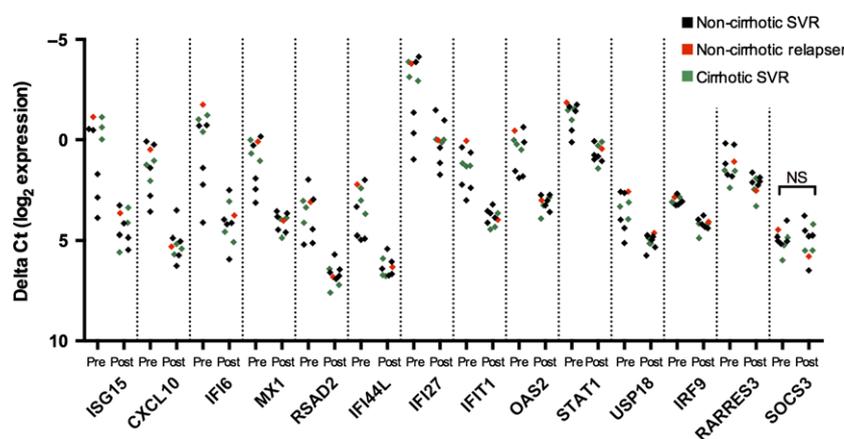


**Fig. 2** IFN-stimulated gene (ISG) expression is downregulated irrespective of cirrhosis. Select ISGs were chosen to compare relative gene expression in noncirrhotic (shown in black, $n = 5$) vs cirrhotic subjects (shown in green, $n = 3$) achieving sustained virologic response. Data from the subject who relapsed (noncirrhotic) are shown in red ($n = 1$). All shown ISGs had significantly decreased expression by the Wilcoxon paired test ($P < 0.05$) over the course of treatment with the exception of SOCS3 (NS = not significant), with no detectable difference based on fibrosis stage. Data from pre- and post-treatment liver biopsies are indicated, with individual genes separated by dashed lines.
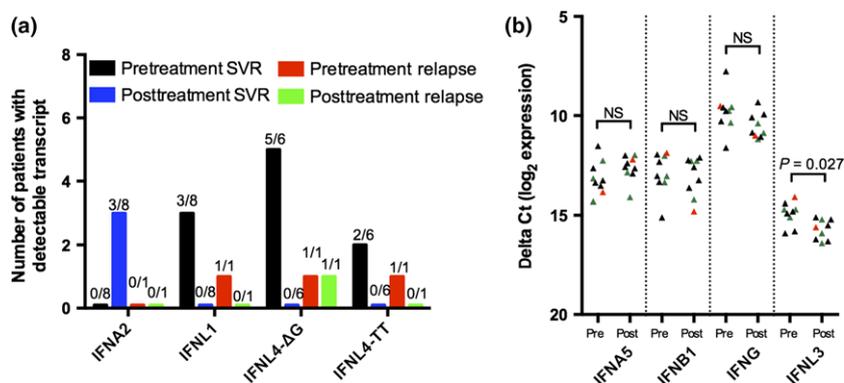
**Fig. 3** Hepatic interferon gene expression changes with treatment. Select interferon genes were amplified from liver mRNA before and after treatment. (**a**) Subjects with detectable *IFNA2* increased during treatment while *IFNL1* and *IFNL4-ΔG* were less frequently detected after treatment. Data are shown as number of subjects with detectable transcript, as defined in the methods, as in many cases transcripts could not be quantified, precluding quantitative statistics. *IFNL4* results are shown for six subjects carrying at least one ΔG allele and from six subjects carrying at least one TT allele (Table 1), with allele specific gene expression displayed. SVR subjects are shown in black/blue and the subject who relapsed in red/green. (**b**) IFNs with quantifiable pre- and post-treatment expression. *IFNL3* decreases over the course of treatment while *IFNA1/4/6/8/16/17* and *IFNL2* were not detectable. Noncirrhotic SVR subjects are shown in black, cirrhotic SVR subjects are shown in green, and the noncirrhotic relapser is shown in red. Statistical analysis for panel B is by Wilcoxon paired test considering all subjects.

putative miRNA target sites and many miRNAs have been associated with HCV pathogenesis and IFN signalling [28,29]. Nine miRNAs known to have high expression in human liver were among the top 100 most highly expressed miRNAs in our RNA-Seq study (miR-122, 192, 199a/b-3p, 101, let-7a, let-7c, let-7b, let7-f and 99a) [30] (data not shown). miR-122 was only the 4th most abundant species identified in our analysis, in contrast to reports suggesting miR-122 constitutes the majority of miRNA in human liver [30–32]. Importantly, recent work revealed a marked platform-dependent difference in the quantification of miRNA species by RNA-Seq [33]. The level of relative expression we observed for miR-122 and other top miRNAs in our analysis is consistent with other results obtained using the TruSeq platform in HCV liver [34].

Amongst the miRNAs with significant changes over treatment (Table 2), an increase in expression of miR-122 during treatment-induced HCV clearance was the most notable change (1.4 fold increase, $P = 0.034$), with no apparent impact of cirrhosis (Fig. 4). This finding is consistent with reports that miR-122 has lower expression in HCV-infected liver relative to healthy liver and HBV-infected liver [35]. Importantly, endogenous IFN activity may lead to suppression of miR-122 expression, which is an essential cofactor for HCV replication [31]. Interestingly, expression levels of miRNAs induced by IFN-β activity and associated with HCV downregulation *in vitro* (miR-196, miR-296, miR-351, miR-431, miR-448, miR-29, miR-155), as well as expression of miRNAs associated with IFNL regulation (miR-208b, miR-499, miR-508)

[36,37] and endogenous IFN pathway components [28] did not change over the course of therapy in our study.

We did observe upregulation of expression of two miRNAs (miR-30b/30c) previously noted to be downregulated during HCV infection in HUH-7.5 cells, which have been implicated in HCV entry, replication and the host response to infection [38–40]. In contrast, no changes were observed in expression of miR-130a/301, which were upregulated in HCV-subject liver biopsies and have been implicated in HCV suppression *in vitro* [38–40]. Other than miR-122, expression of most miRNAs associated with facilitating or suppressing HCV infection did not change, including several that have target sites within the viral genome (miR-130a, miR-141, miR-21, miR-448, miR-196, miR-let-7b, miR-199a, miR-27a) [41–48]. Finally, expression of miRNAs previously implicated in HCV and fibrosis (miR-107, miR-200, miR-21, miR-29a, miR-155 and miR-449a) [41,42,49] did not change in expression during treatment, nor did we observe differences in expression of these miRNAs based on cirrhosis (data not shown).

## DISCUSSION

With an aim of understanding whether innate immunity could influence higher relapse rates seen in some DAA studies of cirrhotics, we used liver biopsies from before and after IFN-free, RBV-free DAA therapy and examined the impact of cirrhosis on changes in endogenous IFN pathways during treatment. In contrast to our expectations, we observed similar changes in hepatic expression of genes associated with IFN signalling irrespective of liver cirrhosis

**Table 2** miRNAs that change significantly over the course of treatment

| miRNA | Mean (pre) | Mean (post) | Fold change | *q*-Value |
|---|---|---|---|---|
| hsa-miR-122-5p | 1386.8 | 1935.4 | 1.40 | 0.034 |
| hsa-miR-26a-5p | 1210.7 | 1786.7 | 1.48 | 0.043 |
| hsa-miR-26a-5p | 1192.2 | 1777.9 | 1.49 | 0.038 |
| hsa-miR-423-5p | 845.5 | 718.4 | -1.18 | 0.046 |
| hsa-miR-148a-3p | 842.7 | 1608.6 | 1.91 | 0.029 |
| hsa-miR-30d-5p | 573.2 | 777.3 | 1.36 | 0.029 |
| hsa-miR-191-5p | 554.7 | 713.0 | 1.29 | 0.032 |
| hsa-miR-26b-5p | 137.9 | 192.7 | 1.40 | 0.039 |
| hsa-miR-28-3p | 131.1 | 157.5 | 1.20 | 0.034 |
| hsa-miR-378a-3p | 82.8 | 153.9 | 1.86 | 0.021 |
| hsa-miR-194-5p | 69.7 | 100.0 | 1.44 | 0.046 |
| hsa-miR-375 | 66.8 | 117.8 | 1.76 | 0.029 |
| hsa-miR-30c-5p | 64.5 | 92.9 | 1.44 | 0.040 |
| hsa-miR-194-5p | 59.4 | 86.4 | 1.45 | 0.044 |
| hsa-miR-30b-5p | 48.3 | 74.0 | 1.53 | 0.029 |
| hsa-miR-23b-3p | 46.7 | 94.6 | 2.03 | 0.029 |
| hsa-miR-151a-3p | 43.7 | 53.9 | 1.23 | 0.029 |
| hsa-miR-103a-3p | 36.4 | 43.6 | 1.20 | 0.039 |
| hsa-miR-151a-5p | 32.3 | 40.1 | 1.24 | 0.034 |
| hsa-miR-93-5p | 24.3 | 29.4 | 1.21 | 0.029 |
| hsa-miR-574-3p | 17.5 | 25.2 | 1.44 | 0.029 |
| hsa-miR-200b-3p | 16.0 | 21.0 | 1.31 | 0.039 |
| hsa-miR-193b-3p | 15.2 | 23.4 | 1.53 | 0.034 |
| hsa-miR-21-3p | 12.5 | 8.4 | −1.49 | 0.039 |
| hsa-miR-148a-5p | 12.0 | 19.1 | 1.59 | 0.029 |
| hsa-miR-361-5p | 9.0 | 12.4 | 1.38 | 0.029 |
| hsa-miR-125b-1-3p | 8.4 | 5.8 | −1.46 | 0.043 |
| hsa-miR-885-5p | 6.2 | 8.2 | 1.34 | 0.036 |
| hsa-miR-181a-2-3p | 5.7 | 9.9 | 1.75 | 0.029 |
| hsa-miR-320b | 4.4 | 3.2 | −1.38 | 0.029 |
| hsa-miR-455-3p | 3.6 | 6.6 | 1.87 | 0.029 |
| hsa-miR-20a-5p | 2.9 | 4.5 | 1.53 | 0.034 |
| hsa-miR-194-3p | 2.8 | 3.8 | 1.37 | 0.044 |
| hsa-miR-877-5p | 2.4 | 1.4 | −1.80 | 0.038 |
| hsa-miR-421 | 2.4 | 3.7 | 1.54 | 0.038 |
| hsa-miR-30c-2-3p | 2.4 | 3.4 | 1.44 | 0.034 |
| hsa-miR-365b-3p | 1.8 | 3.1 | 1.77 | 0.029 |
| hsa-miR-505-3p | 1.4 | 2.8 | 1.97 | 0.030 |
| hsa-miR-3909 | 1.3 | 1.9 | 1.49 | 0.045 |

Shown are mean pre- and post-treatment expression counts, relative fold change over the course of treatment and *q*-values (FDR-corrected *P*-values) as described in the methods section. Results are organized based on highest pre-treatment expression counts.

or the use of RBV. Our results demonstrate distinct down-regulation of type III IFNs during DAA treatment and upregulation of select type I IFNs in the liver of some subjects who achieve SVR, in support of previous observations [12]. Thus, changes in endogenous IFNs seem to occur regardless of treatment duration, stage of liver disease, presence of RBV or use of specific DAA regimen. As all cirrhotic subjects examined here achieved SVR, we cannot conclude that cirrhotic subjects who relapsed in other trials do not have defects in intrahepatic innate immune reconstitution.

The sole individual who relapsed in this study had detectable expression of *IFNL4-ΔG* transcript at the end of treatment, similar to the subject with paired liver biopsies who relapsed after SOF/RBV treatment in the SPARE trial [12]. Persistent *IFNL4-ΔG* expression in post-treatment liver of both subjects who relapsed in independent liver biopsy studies is intriguing, as only 1 of 12 subjects with
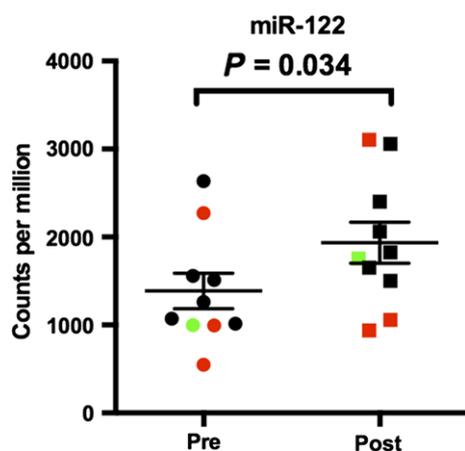
**Fig. 4** Changes in miR-122 expression over the course of treatment. Shown are RNA-Seq data derived from analysis of paired liver biopsy RNA using the Illumina TruSeq platform. $N = 10$ subjects, with three cirrhotic subjects shown in red and one subject who relapsed shown in green. Pre- and post-treatment means with standard error are shown. Statistical analysis is by paired *t*-test with a multiple-test correction, as described in the methods.

paired biopsies in these studies who achieved SVR and who carry an *IFNL4-ΔG* allele had detectable *IFNL4-ΔG* expression at post-treatment liver [12]. Expression of IFNL4 protein has been reported to have pathogenic effects in human hepatic cells [50], and another report has associated *IFNL4-ΔG* expression with intrahepatic ISG expression in chronic infection [13]. It is thus interesting to speculate that post-treatment *IFNL4-ΔG* expression could have prognostic value as a measure of a sustained immune response to persistent virus after treatment in subjects that may eventually experience treatment relapse. This suggestion is tempered by the absence of a difference in *IFNL4-ΔG* expression in unpaired, post-treatment biopsies between subjects who achieved SVR *vs* those who relapsed after SOF/RBV treatment [12].

Interestingly, we did not observe downregulation of *IFNG* transcript over the course of treatment (Fig. 3), in contrast to the significant decrease in *IFNG* observed in subjects treated with SOF/RBV for 24 weeks. While this may be attributable to the small sample size of our study, it suggests a potential role for RBV in modulating *IFNG* gene expression. Alternatively, a longer duration of treatment may be required for *IFNG* downregulation, as subjects in SYNERGY were treated for 6–12 weeks as compared to 24 weeks in SPARE, and HAI inflammatory score did not decrease in 3 of 6 subjects treated with a 6-week regimen here.

As in our previous study, induction of *IFNA2* was observed in a subset of subjects who achieved SVR. Here, we extended our analysis to other type I IFNs, but found most could not be detected in liver with the sensitivity of the assay and the amount of material available for analysis.

Unchanged gene expression of *IFNB1*, other detectable type I IFNs, and *IFNG* over the course of treatment all lend support to the opinion that type III IFNs play a prominent role in driving aberrant ISG expression in chronic HCV infection.

Finally, we were surprised that expression levels of multiple miRNA species previously implicated in HCV replication and endogenous IFN signalling did not change over the course of treatment. Importantly, marked differences in miRNA expression have been observed depending on the platform used for analysis [33], and as such, direct comparison of data generated using the TruSeq platform in our study may be problematic. Furthermore, many HCV miRNA studies were conducted *in vitro*, and thus may have less relevance when measuring levels directly from liver tissue. Transcriptional analysis of mRNA and miRNA in the whole-liver samples may be sensitive to changes in the relative composition of liver cells that occurs over the course of treatment, and HAI inflammation, representing immune cell prevalence in liver, declines with treatment in most patients. As changes in specific cellular subsets in HCV liver after DAA treatment have not yet been characterized, the relative contribution of hepatocytes to the mRNA and miRNA signatures discussed here are unknown, but might be expected to increase over the course of treatment. Together, these observations suggest the impact of miRNA regulation in HCV suppression with DAAs *in vivo* requires further analysis.

Several additional limitations of the study may impact the suggested conclusions. Most importantly, as all cirrhotic subjects in this study achieved SVR, we were unable to directly correlate transcriptional findings with the question of how treatment outcome is impacted by cirrhosis. In addition, all cirrhotic subjects with paired biopsies received 12 weeks of therapy and were compared primarily with subjects treated with 6 weeks of therapy. Future studies would be needed to assess whether differences in kinetic changes of on-treatment HCV decline in liver and serum, and hepatic endogenous IFNs, occur with similar treatment durations in cirrhotics and noncirrhotics. Finally, our conclusions regarding the relative contribution of RBV to endogenous IFN signalling are drawn through inference, and to assess an impact directly, paired biopsies from subjects receiving identical DAA regimens with and without RBV would be required.

In conclusion, these data reveal consistent changes in gene expression of endogenous IFN pathways during DAA-mediated HCV suppression irrespective of cirrhosis or use of RBV in this trial in which most subjects achieved SVR. These data confirm the association of type III IFN expression with ISGs in chronic HCV infection, the downregulation of type III family members with DAA-induced HCV expression, and the induction of *IFNA2* gene expression in some subjects achieving SVR. Favourable treatment outcome with DAA therapy for HCV infection correlates with changes in endogenous IFN signalling, suggesting that

host immunity may plan an active role in concert with medication-induced HCV suppression to eradicate virus.

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