Journal of Neuroimmunology 335 (2019) 577022

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Relationships among neurotransmitters, cytokines and cognitive performance for individuals with hepatitis C achieving sustained virologic response: A pilot study

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ARTICLE INFO

Keywords:
Memory
Executive function
Cognitive performance
Cytokines
Hepatitis C

ABSTRACT

An important extrahepatic consequence of Hepatitis C is its adverse impact on the central nervous system and cognitive performance. We aimed to determine whether there is a significant relationship between selected neurotransmitters and cytokines and cognitive performance in patients with Chronic Hepatitis C before and after achieving sustained virologic response (SVR). Pre-SVR, elevated kynurenine was associated with increased immediate and delayed visual memory, whereas post-SVR the positive associations are between kynurenine and immediate and delayed verbal memory. TGF-B was consistently negatively associated with both immediate and delayed visual memory pre- and post-SVR. These concomitant changes may have important clinical relevance.

1. Introduction

Hepatitis C Virus (HCV) infection affects an estimated 64–103 million people worldwide and is considered a major health and economic burden to many countries (Gower et al., 2014). HCV infection is a systemic disease that may lead to both hepatic manifestations (e.g. cirrhosis and hepatocellular carcinoma) and extrahepatic manifestations involving multiple organ systems (e.g. integumentary, ocular, muscular, skeletal, nervous, endocrine, cardiovascular, respiratory, and urinary systems) (Gill et al., 2016). A particularly important extrahepatic consequence of HCV is its adverse impact on the central nervous system and cognitive performance (Cacoub et al., 2014; Fletcher and McKeating, 2012; Younossi et al., 2016). In fact, even after controlling for substance abuse, affective disorders, and cirrhosis, presence of HCV viremia is associated with neurocognitive impairments (Fletcher and McKeating, 2012; Monaco et al., 2012). About one-third of individuals with chronic HCV have decreased levels of cognitive performance (Hilsabeck et al., 2002, 2003), with specific deficits noted in attention, processing speed, working memory, verbal learning, mental flexibility, and problem solving (Huckans et al., 2009; Perry et al., 2008).

However, little is known about the temporal aspects of cognitive performance in people with HCV. In particular, there is little known about what happens after successful treatment of viremia. Additionally, contributors to this change in cognition have not been well characterized, specifically abnormalities in the regulation of metabolism and/or inflammation. Potential contributors may include neurotransmitter and cytokine dysregulation that is associated with prolonged immune system activation (McAfoose and Baune, 2009).

Current treatments for Chronic Hepatitis C (CHC) include direct-acting antivirals, which have been shown to achieve sustained virologic response (SVR) in 90% or more of patients (Kiser et al., 2013; Monaco et al., 2015). A systematic review found that HCV eradication leads to improved cognitive functioning, however, patients who attained SVR were still impaired compared with healthy, age matched individuals (Spiegel et al., 2005). More recent studies have confirmed modest improvements in functioning in individuals who have attained SVR post-treatment compared with individual baselines and to treatment non-responders (Kuhn et al., 2017; Younossi et al., 2017). Overall, these results indicate that SVR decreases impairments but does not result in

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https://doi.org/10.1016/j.jneuroim.2019.577022
Received 5 July 2019; Received in revised form 13 August 2019; Accepted 13 August 2019

return to baseline levels of functioning in all individuals with HCV. Therefore, it is important to understand the potential relationship between serum neurotransmitters and cytokines and cognitive performance in individuals with HCV as potential treatment targets for future investigation. In this pilot study, we aim to investigate the relationship between cognitive performance and serum levels of selected neurotransmitters and cytokines before and after achieving SVR in patients with CHC.

2. Methods

Clinical, cognitive performance, and serum data were utilized from an HCV-eradication multi-center clinical trial assessing efficacy of Ledipasvir (LDV)/Sofosbuvir (SOF). Treatment consisted of a fixed-dose combination (90LDV/400SOF mg). HCV subjects without cirrhosis received 12 weeks of LDV/SOF (Arm 1) or placebo, then treated with 12 weeks of LDV/SOF (Arm 2). The treatment that the placebo group received after completing the placebo time period, was exactly the same as for participants in Arm 1. For the current investigation, both Arm 1 and 2 were combined since we were interested in the relationship between neurotransmitters and cytokines before and after achieving SVR. The time points used were "baseline" – which was defined as the data point closest to the beginning of active treatment and 24-weeks after the completion of treatment. For the group that started in the placebo group, the "baseline" timepoint was after the completion of placebo (12 weeks) and unblinding had occurred, which was the 4-weeks post-placebo trial timepoint. The placebo group received the exact same treatment as Arm 1 and the data points were comparable between the groups. Participants were between the ages of 18–60 and had been diagnosed with CHC for at least six months. Potential participants were excluded from the study if they had clinically significant illnesses, such as hepatic decompensation, significant pulmonary or cardiac disease, HIV, hepatitis B, or cirrhosis. The original clinical trial was approved by the institutional review board or independent ethics committee at each participating clinical site and was conducted in compliance with Good Clinical Practice guidelines and local regulatory requirements. This study was approved by the Inova Fairfax Hospital Institutional Review Board.

2.1. Analyte measurement

Pre- (baseline) and post-treatment (24-weeks post-active treatment; SVR24) serum collected from HCV patients was used to measure circulating analytes ($N = 40$ patients). Laboratory measures with diagnostic value (i.e., ALT, AST, circulating lipids) were measured as standard of care by normal methods. Tryptophan, gamma-aminobutyric acid (GABA), and kynurenine were measured by ELISA (Rocky Mountain Diagnostics; Colorado Springs, CO), as were serotonin (Enzo Life Sciences; Farmingdale, NY), brain-derived neurotrophic factor (BDNF), IFN (R&D Systems; Minneapolis, MN), and catechol-*O*-methyltransferase (COMT), indoleamine 2,3-dioxygenase (IDO), transforming growth factor beta (TGFb) (Life Span Biosciences; Seattle, WA). Chemokine ligand 2 (CCL2), chemokine ligand 3 (CCL3), platelet-derived growth factor (PDGF), and TNF assays were performed as a BioPlex customized assay (BioRad; Hercules, CA). All assays were performed as per manufacturers' instructions.

2.2. Cognitive performance testing

Participants were administered a battery of cognitive performance tests selected from the following validated assessments: Wechsler Adult Intelligence Scale (WAIS-IV) (Carlozzi et al., 2015), Wechsler Memory Scale (WMS-IV) (Carlozzi et al., 2013), Wechsler Test of Adult Reading (WTAR) (Whitney et al., 2010), Hopkins Verbal Learning Test- Revised (HVLT-R) (Benedict et al., 1998), Brief Visuospatial Memory Test- Revised (BVMT-R) (de Caneda et al., 2018), and Delis Kaplan Executive Function System (D-KEFS) (Delis et al., 2001, 2004). Individual subtests were selected based on ability to measure the following cognitive functions: verbal premorbid intelligence, verbal memory (immediate and delayed), visual memory (immediate and delayed), verbal fluency (phonemic and semantic), processing speed, conceptual shifting, inhibition and cognitive flexibility, working memory, and motor speed (Table 1). These subtests have been used to identify and track subtle cognitive performance deficits in several medical conditions, including HIV (Carey et al., 2004), dementia (Kuslansky et al., 2004), traumatic brain injury, and stroke (Muir et al., 2015).

Table 1
Battery of cognitive performance sub-tests administered.

Subtests	Cognitive domain assessed
Wechsler Test of Adult Reading	Verbal premorbid intelligence
Hopkins Verbal Learning Test- Revised	Verbal memory (immediate and delayed)
Brief Visuospatial Memory Test-Revised	Visual memory (immediate and delayed)
FAS Fluency	Verbal fluency (phonemic)
Animal Naming	Verbal fluency (semantic)
Symbol Search (WAIS-IV)	Processing speed
Trail Making Test A	Processing speed
Trail Making Test B	Conceptual shifting
Color-Word Interference Test (D-KEFS)	Inhibition and cognitive flexibility
Digit Span (WAIS-IV)	Working memory
Symbol Span (WMS-IV)	Working memory
Grooved Pegboard	Motor speed

WAIS-IV: Wechsler Adult Intelligence Scale; WMS-IV: Wechsler Memory Scale; D-KFES: Delis Kaplan Executive Function System.

Specifically, the following subtests were used:

- The Wechsler Test of Adult Reading (WTAR): yields a summary score indicative of verbal premorbid intelligence. The examinee is presented with a list of words that range from simple, high frequency words to complex, low frequency words. Performance is based on accuracy of pronunciation, which correlates highly with familiarity and intelligence, particularly verbal intelligence (Whitney et al., 2010). Higher scores indicate better performance.
- Hopkins Verbal Learning Test- Revised (HVLT-R): yields two summary scores: immediate verbal memory and delayed verbal memory. The examinee is presented orally with a list of 10 nouns that come from 3 different semantic categories and they are asked to verbally repeat as many words as they can. This is repeated for two additional trials and is followed by a delayed recall trial (given after 20–25 min) and recognition trial (Bailey et al., 2018; Woods et al., 2005). Immediate verbal memory score is the total amount of words repeated correctly across the three recall trials. Delayed verbal memory score is the total amount of words repeated correctly during the delayed recall trial. Higher scores indicate better performance.
- Brief Visuospatial Memory Test- Revised (BVMT-R): yields two summary scores: immediate visual memory and delayed visual memory. In three learning trials, the examinee views a stimulus page for 10 s and is asked to draw as many of the figures as possible in their correct location on a page in the response booklet. A delayed recall trial is administered after a 25-minute delay. This is followed by a recognition trial in which the examinee is asked to identify which of 12 figures were included among the original geometric figures (de Caneda et al., 2018). Immediate visual memory score is the total amount of images recalled correctly across the three trials. Delayed visual memory score is the total amount of images recalled correctly during the delayed recall trial. Higher scores indicate better performance.
- FAS Fluency: yields a score indicative of verbal fluency (phonemic). The examinee is asked to produce as many words as possible that begin with a specified letter (F,A,S) during a fixed period of time

(1 min). The total correct is the sum of all admissible words for the three letter trials. Higher scores indicate better performance.

- Animal Naming: yields a summary score indicative of verbal fluency (semantic). The examinee is asked to name as many different types of animals as they can in 1 min. The total correct is the sum of all admissible words for the animal category (Tombaugh et al., 1999). Higher scores indicate better performance.
- Trail Making Test A: yields a summary score indicative of processing speed. The examinee must connect, by making pencil lines, 25 encircled numbers randomly arranged on a page in proper order. The score is expressed in terms of time in seconds to complete (Sánchez-Cubillo et al., 2009). Lower scores reflect better performance.
- Trail Making Test B: yields a summary score indicative of conceptual shifting, relative to overall processing speed (Trail Making Test A). The examinee must connect, by making pencil lines, 25 encircled numbers and letters randomly arranged on a page in proper alternating order. The score is expressed in terms of the time in seconds to complete (Sánchez-Cubillo et al., 2009). Lower scores indicate better performance.
- Grooved Pegboard: yields two summary scores indicative of motor speed: dominant hand and non-dominant hand. It consists of a metal board with a matrix of 25 holes with randomly positioned slots. The examinee is asked to insert pegs as quickly as possible into the slots in sequence, first with the dominant hand and then with the non-dominant one. The scores are expressed in terms of time in seconds to complete the task with each hand (Ruff and Parker, 1993). Lower scores indicate better performance.
- Digit Span: yields two scores indicative of working memory: digit span forward and digit span backward. The examinee is asked to verbally repeat strings of digits of increasing length said by the examiner in the same (forward) and in reverse (backward) order. Both scores are expressed as the sum of correct responses for each forward and backward trial. (Jacola et al., 2014; Jasinski et al., 2011; Wechsler, 2008). Higher scores indicate better performance.
- Symbol Search: yields one score indicative of processing speed. The examinee scans a search group and indicates with a pencil whether one of the symbols in the target group matches the search group. The score is expressed as the total number of incorrect items subtracted by the total number of correctly identified items in a 2 min period (Wechsler, 2008). Higher scores indicate better performance.
- Symbol Span: yields one score indicative of working memory. The examinee is briefly shown a series of abstract symbols on a page for 5 s and then asked to select the symbols from an array of symbols, in the same order they were presented on the previous page. The score is expressed as the total number of symbols recalled correctly and/or in the correct order (Wechsler, 2009). Higher scores indicate better performance.
- Color-Word Interference: yields a summary score indicative of inhibition and cognitive flexibility. The examinee is asked to name color patches (Trial 1), read words that denote colors printed in black ink (Trial 2), inhibit reading words denoting colors in order to name the dissonant ink colors in which those words are printed (Trial 3), and switch back and forth between naming the dissonant ink colors and reading the conflicting words (Trial 4) (Delis et al., 2001). Trial scores are expressed as time (in seconds) to complete. Lower scores indicate better performance.

2.3. Statistical analysis

Clinico-demographic parameters, cognitive performance test scores and measured cytokines and neurotransmitters were summarized as N (%) or mean ± standard deviation for all enrolled patients as a group. The minimal clinically important difference (MCID) was calculated by computing 5% of the total range size for an outcome. For example, for an outcome that has potential scores that range from 80 to 320, 12 is 5% of that range. We considered a 12-point change as the MCID for that outcome. Similar quantitative calculations have been used to calculate MCID (Norman et al., 2003). Correlations of cognitive performance scores with neurotransmitters and cytokines and changes in those were calculated using Spearman's non-parametric method. All analyses were run in SAS 9.4 (SAS Institute, Cary, NC).

Table 2
Clinico-demographic characteristics of patient population.

N	40
Age (mean ± std.dev.)	45.3 ± 11.5
Male, n (%)	19 (47.5%)
Body Mass Index (kg/m^2)	25.9 ± 3.6
Race, n (%)	
White	36 (90.0%)
Black	4 (10.0%)
Employed, n (%)	35 (89.7%)
Treatment NAÏVE	23 (57.5%)
Laboratory measurements:	
ALT (U/l)	58.6 ± 34.0
AST (U/l)	43.3 ± 20.3
Bilirubin (mg/dL)	0.6 ± 0.3
Hemoglobin (g/dL)	14.3 ± 1.3
Elevated liver enzymes	13 (32.5%)
Comorbidities:	
Anxiety	6 (15.0%)
Depression	5 (12.5%)
Fatigue	4 (10.0%)
Diabetes	2 (5.0%)
Achieved SVR	38 (97.4%)

Data are means ± standard deviation or n (%).
ALT: alanine aminotransferase; AST: aspartate aminotransferase, SVR: sustained virologic response.

3. Results

Clinico-demographic parameters are presented in Table 2. The study population was 47.5% males, 90% White with a mean age of 45.3 years ± 11.5. Thirty-eight of the forty included participants reached SVR and were therefore included in analyses. One of those individuals did not participate in the post-treatment visit and was therefore not included in analyses that utilized that data point. There were 37 participants with complete data for analyses.

3.1. Cognitive performance and serum analytes at baseline

Prior to beginning treatment, there were statistically significant relationships present at baseline (Table 3). Visual memory (both immediate and delayed) was related to kynurenine (rho = 0.32, $P = .04$ and rho = 0.31, $P = .05$) and TGF-B (rho = −0.36, $P = .02$ and rho = −0.37, P = .02), while immediate verbal memory was related to TNF (rho = 0.46, $P < .01$). In addition, semantic verbal fluency was related to BDNF (rho = 0.37; P = .02), and both processing speed and motor performance were related to CCL2 (rho = −0.39, P = .02 and

Table 3
Statistically significant correlations between cognitive performance and analytes prior to treatment (baseline).

Cognitive domain	Analyte	Correlation (r)	*p*-Value
Visual memory (immediate)	Kynurenine	0.32	0.04
	TGF-B	−0.36	0.02
Visual memory (delayed)	Kynurenine	0.31	0.05
	TGF-B	−0.37	0.02
Verbal fluency (semantic)	BDNF	0.37	0.02
Verbal memory (immediate)	TNF	0.46	< 0.01
Motor performance	CCL2	0.32	0.05
Processing speed		0.39	0.02

TGF-B: transforming growth factor beta; BDNF: brain-derived neurotrophic factor; TNF: tumor necrosis factor; CCL2: chemokine ligand 2.

Table 4
Cognitive performance at baseline and after achieving sustained virologic response.

Cognitive domain	Baseline	SVR24	*p*-Value
Visual memory (immediate)	50.8 ± 13.1	44.2 ± 13.3	< 0.01
Visual memory (delayed)	51.8 ± 11.1	46.6 ± 12.6	< 0.01
Working memory	10.8 ± 2.9	11.4 ± 3.4	0.14
Verbal fluency (semantic)	22.3 ± 5.0	22.4 ± 5.5	0.35
Verbal memory (immediate)	49.7 ± 9.9	49.1 ± 11.1	0.77
Verbal memory (delayed)	49.6 ± 10.5	50.5 ± 10.6	0.78
Processing speed	10.5 ± 2.8	12.4 ± 3.2	< 0.01
Motor performance	141.2 ± 20.5	133.2 ± 18.0	< 0.01

SVR24: 24-weeks post-active treatment.

rho = 0.32, P = .05).

3.2. Cognitive performance and serum analytes at SVR24

Cognitive performance changes (baseline compared to SVR 24) are presented in Table 4. Then the correlation between cognitive performance and serum analytes were investigated at twenty-four weeks post-treatment after achieving SVR (Table 5). Verbal memory (both immediate and delayed) was related to kynurenine (rho = 0.44, P < .01 and rho = 0.35, P = .03). In addition, TGF-B was related to immediate and delayed visual memory (rho = −0.36, P = .03 and rho = −0.34, P = .04), while GABA was related to delayed visual memory (rho = 0.46, P < .01). CCL2 was still related to processing speed (rho = −0.35, P = .04) but no longer related to motor speed. Kynurenine and TNF were also both related to processing speed (rho = −0.32, P = .05 and rho = −0.40, P = .02). In addition, COMT was related to working memory (−0.43, P < .01) and immediate visual memory (rho = −0.37, P = .02).

3.3. Relationship between serum analytes and MCID in cognitive performance after sustained virologic response

For the cognitive performance subtests, there were no serum analytes that were related to MCID. However, there were relationships that emerged when examining the combination of subtests into domains. For the working memory summary score (combination of forward digit span, backward digit span, and symbol span), ten individuals demonstrated improved working memory to a MCID (27 individuals that did not). The factors that differentiated those that improved from those that did not were higher baseline levels of TNF (8.58 ± 6.88 vs. 3.96 ± 3.76, P = .02) and lower baseline levels of COMT (0.28 ± 0.25 vs. 0.47 ± 0.30, P = .03). In addition, for the combination of memory tests (immediate and delayed visual memory and immediate and delayed verbal memory), seven individuals improved by a MCID (30 individuals that did not) and they had higher levels of IFN at baseline (3.77 ± 3.38 vs. 1.06 ± 3.27, P = .01), lower levels of COMT at SVR24 (0.25 ± 0.11 vs. 0.47 ± 0.34, P = .04), and higher levels of IFN at SVR24 (3.11 ± 3.23 vs. 1.00 ± 2.18, P = .04). For the conceptual shifting and inhibition and cognitive flexibility summary score (combination of Trail Making Test B, and Color-Word Interference Test), the ten individuals that demonstrated improved performance to a MCID (27 individuals that did not) had higher baseline levels of CCL2 (36.58 ± 9.97 vs. 25.24 ± 15.02, P = .01) and CCL3 (2.23 ± 2.05 vs. 0.82 ± 0.69, P = .01).

Table 5
Statistically significant correlations between cognitive performance and analytes after achieving sustained virologic response.

Cognitive domain	Analyte	Correlation (r)	*p*-Value
Visual memory (immediate)	COMT	−0.37	0.02
	TGF-B	−0.36	0.03
Visual memory (delayed)	GABA	0.46	< 0.01
	TGF-B	−0.34	0.04
Working memory	COMT	−0.43	< 0.01
Processing speed	Kynurenine	−0.32	0.05
	CCL2	−0.35	0.04
	TNF	−0.40	0.02
Verbal memory (immediate)	Kynurenine	0.44	< 0.01
Verbal memory (delayed)		0.35	0.03

COMT: catechol-*O*-methyltransferase; TGF-B: transforming growth factor beta; GABA: gamma-aminobutyric acid; CCL2: chemokine ligand 2; TNF: tumor necrosis factor.

4. Discussion

HCV is a major public health concern worldwide with significant future health and economic burdens predicted. Patients with HCV often complain of various cognitive problems (Hilsabeck et al., 2003). Recently, investigators have begun to explore a possible link between cognitive performance and serum analytes. The purpose of the current pilot study was to examine the relationship between cognitive performance and serum neurotransmitters and cytokines, both in the presence of viremia and after SVR had been achieved.

Our results demonstrate that there are relationships between serum measures of specific analytes and cognitive performance. The most consistent relationships pre and post SVR were with visual and verbal memory performance, the neurotransmitter kynurenine, and the growth factor TGF-B.

Interestingly, pre-SVR, elevated kynurenine was associated with increased immediate and delayed visual memory (Table 3), whereas post-SVR the positive associations are between kynurenine and immediate and delayed verbal memory (Table 5). There is an extensive literature on the roles of the kynurenine pathway in memory formation of model organisms; however, human studies, like this one, are often limited to showing associations of peripheral measurements and memory within the context of specific diseases, such as schizophrenia, bioplar disorder (Platzer et al., 2017), cardiopulmonary bypass recovery, Alzheimer's disease (Giil et al., 2017), and depression (Young et al., 2016). Additionally, these and other studies have shown that kynurenine metabolism is, at least partially, regulated by inflammation associated signaling. Specifically, the kynurenine pathway converts kynurenine primarily to anthranilic acid which is then oxidized to 3-hydrozyanthranilic acid (3HAA). However, in the presence of immune stimulation, the ratio of anthranilic acid to 3HAA is changed, with less anthranilic acid being oxidized. In our investigation, individuals with HCV are going to have immune stimulation caused by the infection. For those that achieve SVR, the immune stimulation may be lessened, which may restore the ratio of anthranilic acid to 3HAA. Future research should investigate the relation of this ratio to cognitive performance.

To our knowledge, these data are the first to associate kynurenine with memory formation in viral hepatitis patients; however, further study, specifically measurement of downstream analytes such as kynurenic acid, 3-OH-kynurenine, and quinolinic acid would be needed to fully investigate this potential pathway.

The negative association between circulating TGF-b and both immediate and delayed visual memory is consistent pre-and post-SVR. Because circulating TGF-b may positively correlate in some patients with the degree of hepatic fibrosis, the relationship between memory and TGF-b may be indirect. However, TGF-b proteins are multifunctional cytokines whose neural functions are increasingly being recognized. TGF-b signaling is present in the central nervous system and there is evidence of its involvement in the development and plasticity of the nervous system. Therefore, it is feasible that this factor is implicated in changes in cognitive performance seen in the presence and absence of HCV. TGFb is potentially important to understanding the changes in cognitive performance that were identified in the current investigation.

This study does have some limitations. First, there was only a 24-

week follow-up, which is relatively short. A longer period between SVR and follow-up will be important to examine. Analytes chosen for this study were surrogates for pathways that have previously been implicated in cognitive performance. However, to hypothesize about the role or mechanism of the specific analytes without more in-depth investigation of upstream and downstream members of the relevant pathways would be premature. This study was a correlative study which prohibits inferences about causality and does not rule out the possibility of other unidentified variables that may account for or modify the reported associations. In addition, the range of cognitive performance tests was limited, a broader approach to cognitive performance in future research will be important. Finally, this investigation utilized repeated cognitive performance testing. There might have been a learning effect since the participants were completing the tasks more than once. Since our investigation has a one-group pre-post design, we are not able to assess the potential learning effect. However, it is important to note that not all participants showed an improvement in cognitive performance testing scores, which may be evidence that the learning effect may not be a strong factor in this investigation.

5. Conclusions

In conclusion, our pilot study shows that the relationships between cognitive performance and serum analytes were different at baseline and after achieving SVR24. This suggests that the relationships between cognitive performance and circulating analytes can change with viral eradication. In addition, it adds to the growing literature implicating chronic immune activation as a contributor to cognitive performance decrements (Hilsabeck et al., 2010), raising the prospect of novel targets for interventions. Future studies are needed to better understand the underlying mechanisms associating these serum analytes to cognitive performance, with the long-term goal of investigating the mechanisms by which cognitive function improves following clearance of HCV.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The clinical trial in which data from this study was collected was funded by Gilead Sciences. Data collection, analysis, and manuscript preparation was supported by the Betty and Guy Beatty Center for Integrated Research.

Declaration of Competing Interest

Ali A. Weinstein- No conflict.
J. Michael Estep- No conflict.
Leyla de Avila- No conflict.
Michael Curry- No conflict.
Pegah Golabi- No conflict.
Carey Escheik- No conflict.
Aybike Birerdinc- No conflict.
Maria Stepanova- No conflict.
Jillian K. Price- No conflict.
Lynn Gerber- No conflict.
Zobair M. Younossi- is a consultant to BMS, Gilead, AbbVie, Intercept, and GSK.

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