Hepatol Int
DOI 10.1007/s12072-011-9291-x

ORIGINAL ARTICLE

Serum apolipoprotein C-III is independently associated with chronic hepatitis C infection and advanced fibrosis

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Received: 19 January 2011 / Accepted: 16 June 2011

Abstract
Background The hepatitis C virus (HCV) is known to disrupt lipid metabolism, making serum lipoprotein levels good candidates to explore as markers of HCV disease progression. Assessment of the major apolipoproteins (Apo) and their relationship to hepatic fibrosis remain largely unexplored.
Methods We compared the levels of total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), and Apo A-I, -B, -C-III, and -E between patients with cleared versus active infection ($n = 83$), and between those chronically infected patients ($n = 216$) with advanced versus mild–moderate hepatic fibrosis (METAVIR stage F3–4 vs. F0–2) using multiple logistic regression.
Results Apo C-III levels were 25% higher in subjects with cleared infection versus those with active infection ($p = 0.009$). Low levels of Apo C-III ($p = 1.3 \times 10^{-5}$), Apo A-I ($p = 2.9 \times 10^{-5}$), total cholesterol ($p = 5.0 \times 10^{-4}$), LDL-C ($p = 0.005$), and HDL-C ($p = 2.0 \times 10^{-4}$) were associated with advanced fibrosis in univariate analyses. Multivariable analysis revealed Apo C-III as the most significant factor associated with advanced fibrosis ($p = 0.0004$), followed by age ($p = 0.013$) and Apo A-I ($p = 0.022$). Inclusion of both Apo C-III and Apo A-I in a model to predict advanced fibrosis improved the area under the receiver operator curve only modestly.
Conclusions Relative to other lipoproteins, low serum Apo C-III levels are the most strongly associated with chronic versus cleared infection and decline with increasing severity of hepatic fibrosis. Apo C-III deserves further attention as a possible marker of HCV disease progression.

Keywords Apolipoprotein C-III · Apolipoprotein AI · Hepatitis C virus · Fibrosis

Introduction

Chronic hepatitis C (CHC) infection is a global health problem with estimates of 120–130 million infected persons worldwide [1]. The hepatitis C virus (HCV) has been noted to mediate effects on lipid metabolism by impairing very low-density lipoprotein (VLDL) hepatic assembly and secretion [2, 3]. CHC infection has been most consistently associated with reduced total cholesterol, low-density lipoprotein cholesterol (LDL-C), and apolipoprotein (Apo) B levels [4–6]. This disruption of lipid metabolism may lead to an imbalance favoring steatosis and disease progression by increasing lipogenesis and decreasing the secretion and beta-oxidation of lipids. HCV associates with host lipid metabolism pathways for cellular entry, replication, assembly, and secretion. Low lipid levels in CHC have been correlated with steatosis [5, 6] and more

Electronic supplementary material The online version of this article (doi:10.1007/s12072-011-9291-x) contains supplementary material, which is available to authorized users.

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Published online: 07 July 2011

advanced liver fibrosis [7, 8]. HCV genotype-3 infection is associated with "viral" mediated steatosis, lower total cholesterol levels, and higher fibrosis progression rates [9]. In contrast, lipid disturbances and steatosis in HCV genotype-1 infection appear more dependent on host "metabolic" factors such as insulin resistance and obesity [10]. Perhaps, HCV core protein genotype-specific interference with VLDL assembly and secretion may result in lower total cholesterol levels seen in HCV genotype-3 infection [11], but the biological pathways linking host–viral effects on lipid metabolism and fibrogenesis are yet to be clearly defined. There has been a paucity of studies evaluating the association between CHC disease pathogenesis and specific Apo markers for VLDL, such as Apo C-III and Apo E [12]. Given the direct interaction of the HCV core protein on VLDL assembly and secretion, we hypothesized that lipoproteins related to VLDL may be associated with both HCV RNA and disease severity in a genotype-specific manner. Our aims were to evaluate a panel of serum lipoproteins in a single-center chronic HCV cohort and assess for differences in relation to chronic infection, viral clearance, and disease severity.

Materials and methods

Patient population

Our Duke Hepatology Clinical Research Database and Biorepository is an ongoing registry of HCV-infected subjects and represents a large, well-phenotyped collection of chronic HCV patients. The database includes clinical and demographic data from the medical record, laboratory reports, and case-report forms for these patients. For the current study, we selected subjects who had serum samples drawn before and after interferon-based therapy, with available baseline biopsy. Subjects with other chronic liver disease, or coinfected with the hepatitis B virus (HBV) or the human immunodeficiency virus (HIV) were excluded. Our first aim was to determine the effect of chronic infection and viral clearance on lipoprotein profiles. We selected 83 CHC patients with sustained virologic response to therapy (defined as HCV RNA undetectable at 24 weeks posttreatment); 54 patients had serum available prior to treatment (chronic infection) and 29 had serum after successful treatment (cleared infection). For our second aim, to determine the relationship between hepatic fibrosis and a serum lipoprotein profile, samples were selected from 216 CHC patients with pretreatment liver biopsy.

Liver fibrosis was determined by expert histopathologists using the METAVIR scoring system (F0–F4) and coded as a two-level variable (mild–moderate—F0, F1, F2—vs. advanced—F3, F4) for analysis [13]. HCV genotype was determined by the INNOLIPA HCV assay (Innogenetics, Zwijnaarde, Belgium) and classified as genotype 1, 2, 3, and other (which included genotype 4, unknown, unclassified, and mixed genotypes) for analysis. Body mass index (BMI; kg/m^2) was calculated from height and weight measured at enrollment. Race was defined, self-reported, as African American, Caucasian, or other. Frozen non-fasting serum samples were analyzed at a National Heart, Lung, and Blood Institute/Centers for Disease Control and Prevention Lipid Standardization Program certified laboratory (Children's Hospital Boston, MA). Standard lipoproteins including triglycerides, total-, high-, and low-density lipoprotein cholesterol (HDL-C and LDL-C) were measured directly with reagents from Roche Diagnostics (Indianapolis, IN). Apo AI, B, C-III, and E were measured with immunoturbidometric assays (DiaSorin Inc., Stillwater, MN). All patients had provided prior consent and the study protocol was approved by the Duke University Institutional Review Board.

Statistical analysis

All statistical analyses were performed using SAS software, version v.9.13 (SAS Institute, Cary, NC, USA). All lipoprotein traits had a Gaussian distribution except Apo C-III, Apo E, triglycerides, HDL-C, and Apo B, which were normalized with natural log base 2 (ln) transformations for analysis. However, non-ln-transformed values are presented in the tables. Correlations between lipoproteins were measured with a Pearson's correlation coefficient. Quantitative traits were compared using one-way analysis of variance (ANOVA) and discrete traits were compared using logistic regression analysis.

To assess the relationship between lipoprotein values and advanced versus mild–moderate hepatic fibrosis, multivariable logistic regression models were built using covariates showing significant association ($p < 0.05$) in univariable analysis. Colinearity was assessed prior to inclusion of all covariates. In cases where two variables were highly correlated (e.g., HDL-C and Apo A- I levels and LDL-C and total cholesterol levels), only one was included in the final model. Odds ratios are presented as the odds per unit (standard deviation) increase in each trait. The area under the receiver operator curve (AUROC) for predicting advanced fibrosis was calculated from the c-index in SAS. Sensitivity and specificity values were generated from the logistic procedure classification table and cutoff values were picked based on a probability row (range 0–1.0) with the greatest number of correct predictions and highest Youden's Index [sensitivity − (1 − specificity)].

Results

The study population was predominantly male, in their 40s, of Caucasian race, and infected with HCV genotype 1. There were no significant differences between study groups for demographic variables such as gender, race, or HCV genotype, although patients with advanced fibrosis were older with a higher BMI (Tables 1, 2).

The relationship between age, BMI, and lipoprotein measures are shown in supplementary Table 1 and match the expected patterns based on their established biological relationship (Fig. 1). Total cholesterol was highly correlated with the cholesterol-rich lipoprotein LDL-C and its associated Apo B; HDL-C and LDL-C were both strongly correlated with their constituent Apo A-I and Apo B, respectively; Apo C-III, Apo E, and triglycerides were all significantly correlated with each other, consistent with their association in VLDL.

Chronic infection and viral clearance

To determine which lipoproteins changed the most following clearance of HCV infection, we compared subjects with chronic HCV infection to a separate cohort that had

Table 1 Comparison between patients with chronic versus cleared HCV infection

Discrete traits	Chronic ($N = 54$) N (%)	SVR ($N = 29$) N (%)	p value
Sex			0.949
Males	35 (65)	19 (66)	
Females	19 (35)	10 (34)	
Race			0.439
African Americans	9 (17)	3 (10)	
Caucasians	45 (83)	26 (90)	
HCV genotype			0.943
1	32 (59)	20 (69)	
2	13 (24)	6 (21)	
3	4 (7)	3 (10)	
Other	5 (9)	0 (0)	
Fibrosis stage			0.164
0	2 (4)	0 (0)	
1	10 (19)	10 (34)	
2	30 (56)	8 (28)	
3	8 (15)	7 (24)	
4	4 (7)	4 (14)	

Quantitative traits	Unadjusted mean ± SE	Unadjusted mean ± SE	Unadjusted p value	Adjusted mean ± SE	Adjusted mean ± SE	Adjusted p value
Age (years)	46.6 ± 0.8	48.2 ± 1.1	0.265			
BMI (kg/m^2)	27.7 ± 0.7	28.7 ± 0.9	0.374			
Total cholesterol (mg/dL)	183.1 ± 5.8	190.3 ± 7.9	0.461	174.2 ± 9.3	180.9 ± 12.1	0.498
Triglycerides (mg/dL)[a]	127.8 ± 10.2	167.1 ± 14.0	0.059	113.7 ± 17.8	152.2 ± 23.2	0.082
HDL-C (mg/dL)[a]	47.1 ± 2.0	42.4 ± 2.7	0.273	52.2 ± 3.2	48.1 ± 4.1	0.308
Apo A-1 (mg/dL)	136.5 ± 3.2	128.3 ± 4.4	0.137	145.4 ± 5.0	138.3 ± 6.5	0.180
LDL-C (mg/dL)	110.2 ± 5.1	109.6 ± 7.0	0.942	99.4 ± 8.7	97.8 ± 11.4	0.860
Apo B (mg/dL)[a]	81.9 ± 3.2	90.2 ± 4.4	0.146	72.7 ± 5.4	80.0 ± 7.0	0.262
Apo E (mg/dL)[a]	4.4 ± 0.2	4.1 ± 0.2	0.156	4.7 ± 0.3	4.5 ± 0.3	0.201
Apo C-III (mg/dL)[a]	9.9 ± 0.5	12.5 ± 0.7	0.010	9.0 ± 0.8	11.6 ± 1.1	0.009

SE standard error

All adjusted for age, sex, race, HCV genotype, fibrosis score, and BMI

[a] ln (natural log) transformed for analysis

Table 2 Factors associated with advanced liver fibrosis in univariable analysis among 216 chronic HCV patients

Discrete traits	Mild–moderate ($N = 144$) N (%)	Advanced ($N = 72$) N (%)	Odds ratio (95% CI)	p value
Female sex (vs. male)	61 (42)	22 (31)	0.60 (0.33, 1.10)	0.094
African American race (vs. Caucasian)	38 (26)	24 (33)	1.40 (0.76, 2.6)	0.29
HCV genotype				0.79
1	117 (81)	58 (81)	1.00	
2	14 (10)	5 (7)	0.72 (0.25, 2.10)	
3	5 (3)	4 (6)	1.61 (0.42, 6.24)	
Other	8 (6)	5 (7)	1.26 (0.40, 4.02)	
Quantitative traits	Unadjusted Mean ± SD	Unadjusted Mean ± SD		
Age (years)	47.3 ± 8.9	50.5 ± 5.8	1.53 (1.12, 2.10)	0.008
BMI (kg/m^2)	28.4 ± 5.3	30.5 ± 6.4	1.46 (1.08, 1.96)	0.013
Total cholesterol (mg/dL)	181.0 ± 42.8	158.6 ± 42.1	0.57 (0.42, 0.79)	5.0×10^{-4}
LDL-C (mg/dL)	105.4 ± 37.7	90.1 ± 34.2	0.65 (0.48, 0.88)	0.005
Apo B (mg/dL)	80.0 ± 22.7	75.0 ± 24.7	0.81 (0.61, 1.08)	0.14
HDL-C (mg/dL)	49.6 ± 17.0	39.9 ± 15.9	0.52 (0.38, 0.73)	2.0×10^{-4}
Apo A-I (mg/dL)	143.9 ± 27.8	125.0 ± 30.1	0.49 (0.36, 0.69)	2.9×10^{-5}
Apo C-III (mg/dL)[a]	11.0 ± 4.9	8.4 ± 3.2	0.46 (0.32, 0.65)	1.3×10^{-5}
Apo E (mg/dL)[a]	4.6 ± 1.5	4.3 ± 1.3	0.80 (0.60, 1.07)	0.13
Triglycerides (mg/dL)[a]	132.5 ± 70.3	137.8 ± 53.4	1.20 (0.90, 1.60)	0.21

Odd ratio presented as odds per unit (standard deviation)

Mild–moderate, stage F0–2; advanced, stage F3–4

SD standard deviation

[a] ln transformed for analysis

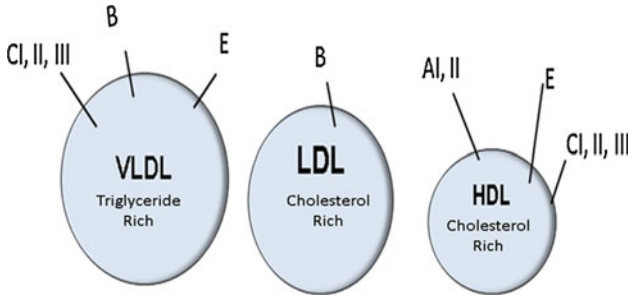

Fig. 1 Partial depiction of the relationship between lipoproteins (very low density, low density, and high density) and apolipoproteins (Apo A, B, C, and E)

successfully achieved SVR following IFN-based treatment. There were no significant differences in demographic traits noted between the chronic and cleared subjects. By ANOVA, the largest difference in means among lipoproteins was seen for Apo C-III, where adjusted mean levels were 25% higher in subjects who cleared virus compared to those who remained chronically infected (Table 1). Total cholesterol, triglyceride, and Apo B levels were marginally higher in the viral clearance group compared to those with chronic infection, but these differences were not significant.

Advanced stage disease

A comparison of subjects with advanced versus mild–moderate fibrosis revealed significantly lower levels of Apo C-III, ApoA-I, total cholesterol, LDL-C, and HDL-C in the former, in univariate analysis (Table 2). There were no associations with gender, race, or viral genotype. As expected, we observed a high correlation between Apo A-I and HDL-C ($r^2 = 0.88$) and total and LDL-C ($r^2 = 0.92$) (supplementary Table 1). Thus, only one of each pair was chosen for inclusion in the multivariable model, so as to avoid statistical errors arising due to multicollinearity. In a multivariable model of advanced fibrosis including age, BMI, LDL-C, Apo A-I, and Apo C-III, the association with Apo A-I was greatly attenuated, and Apo C-III emerged as the most significant factor associated with advanced stage disease ($p = 0.0004$; Table 3). Inclusion of the alternate variable (HDL-C for Apo A-I or total cholesterol for

Table 3 Significant variables associated with advanced liver fibrosis in multivariable analysis

	Odds ratio[a] (95% confidence interval)	*p* value
Age (years)	1.57 (1.10, 2.23)	0.013
BMI (kg/m^2)	1.55 (1.10, 2.19)	0.013
LDL-C (mg/dL)	0.72 (0.51, 1.03)	0.072
Apo A-I (mg/dL)	0.65 (0.45, 0.94)	0.022
Apo C-III (mg/dL)	0.49 (0.33, 0.73)	0.0004

Total cholesterol and HDL-C were excluded due to collinearity with LDL-C and Apo A-1

[a] Odds ratio presented as odds per standard deviation increase in trait

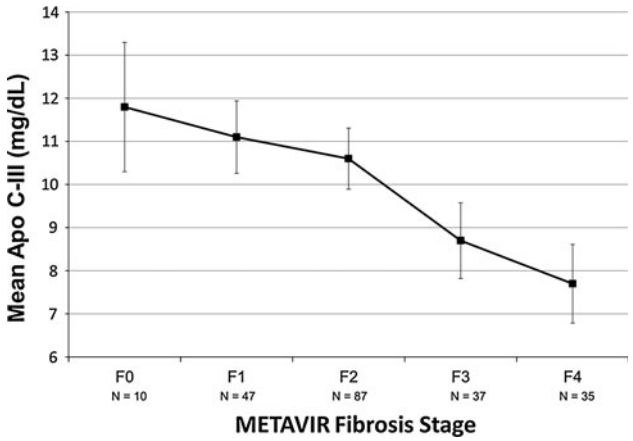

Fig. 2 Mean levels of Apo C-III ± standard error in 216 patients with chronic HCV infection by METAVIR fibrosis stage. Apo C-III levels are adjusted for age, race, sex, HCV genotype, and body mass index

LDL-C) in the model did not alter the findings (data not shown). Figure 2 illustrates how in the final model, the mean adjusted Apo C-III levels significantly decrease with increasing METVIR fibrosis score ($p < 0.0001$).

We next assessed the performance of this model for predicting advanced fibrosis (Table 4). The model that includes Apo C-III has improved sensitivity (47.2 vs. 27.1%), but lower specificity (85.4 vs. 95.1%) compared to the model with Apo A-I. Inclusion of both Apo C-III and Apo A-I in the model improved the AUROC only modestly.

Discussion

HCV infection is known to cause marked alterations in serum lipoprotein levels, but a broad assessment of which lipoproteins are most affected by HCV infection, as well as the clinical relevance of these changes, has not been determined. In our study of various lipoproteins among HCV patients from a US tertiary care single-center cohort, we found that Apo C-III levels were most strongly associated with CHC, with posttreatment levels that were 25% higher in subjects who cleared infection compared to those with chronic infection. In addition, we found that a low level of serum Apo C-III was significantly associated with advanced liver fibrosis.

There is little published data pertaining to Apo C-III levels in the setting of CHC infection. In a previous study, using an unbiased proteomics approach, Apo C-III levels were noted to be significantly lower in French blood donors with CHC compared to blood donors with resolved infection and to those with no history of HCV infection [12]. Apo C-III is synthesized in the liver and intestine and resides mostly on VLDL during the non-fasting state and HDL-C in the fasting state [14, 15]. It is possible that some samples in this study were collected in a non-fasting state and that the total Apo C-III levels measured in this study reflect primarily VLDL-associated Apo C-III. However, while Apo C-III shows the most significant relationship with HCV infection and liver fibrosis in our study, Apo E, another characteristic apolipoprotein for VLDL, does not show a similar association. This may be due to independent transcriptional regulation as Apo E and Apo C-III are attached to VLDL after particle formation with Apo B in the endoplasmic reticulum [16, 17]. In the context of CHC, the transcriptional regulation of Apo C-III may be directly affected by the HCV core protein or indirectly influenced by HCV-associated inflammatory cytokines [18–24]. In addition, low Apo C-III levels in CHC may also reflect a direct perturbation of VLDL metabolism by HCV, which has been noted to directly affect VLDL assembly and secretion. Specifically, HCV core protein inhibits the rate-limiting enzyme (microsomal triglyceride transfer protein) needed for VLDL assembly and the HCV virion is thought to use the VLDL pathway for assembly and secretion [4].

Table 4 Prediction models of advanced fibrosis

Prediction model	AUROC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	–LR
Age, BMI, Apo A-I	0.715	27.1	95.1	73.9	72.5	5.53	0.77
Age, BMI, Apo C-III	0.743	47.2	85.4	61.7	76.4	3.23	0.62
Age, BMI, Apo A-I, and Apo C-III	0.765	48.6	85.4	62.4	76.9	3.33	0.60

PPV positive predictive value, *NPV* negative predictive value, *+LR* positive likelihood ratio, *–LR* negative likelihood ratio

Infectious HCV particles, lipo-viral particles (LVPs), resemble VLDL and are rich in triglycerides, Apo B, and Apo E. Few studies have examined VLDL directly, but many have examined triglycerides, a reasonable surrogate. In the context of standard-of-care therapy with interferon, studies have consistently shown increases in triglycerides and VLDL during treatment with levels returning to baseline after cessation of therapy [25–27]. It is unclear whether this represents removal of HCV-mediated effect on VLDL assembly and secretion or due to interferon treatment itself.

From our cross-sectional data, it is unclear whether Apo C-III levels directly influence disease progression or represent an epiphenomenon. In normal subjects, Apo C-III promotes VLDL production by allowing the escape of the lipoprotein from the hepatocyte surface [14]. VLDL in serum has been shown to inhibit HCV LVPs binding to cell entry receptors on hepatocytes [28]. Thus, one can speculate that low levels of Apo C-III might promote HCV LVP binding to hepatocytes and perhaps increase pathogenicity. On the contrary, Apo C-III may simply be a marker of viral activity via transcriptional downregulation of Apo C-III mediated through PPARα, cytokines, or other potential cell signaling factors effected by the HCV virus or fibrogenesis [18, 20, 21]. Although, it is possible that the low levels of Apo C-III with advanced stage disease simply reflect compromised liver function, this is unlikely given that all patients included in this CHC cohort were being considered for antiviral therapy and required to have compensated liver disease. Some animal studies have linked low Apo C-III levels with hepatic steatosis, but this relationship in humans is not known at this time [29, 30]. Genotype-specific HCV association with Apo C-III and steatosis also requires further study. Prior studies of the association between serum lipoproteins and hepatic fibrosis in CHC infection have found Apo A-I levels to be strongly associated with advanced fibrosis [31]. Indeed, Apo A-I is one of the markers used in the commercially available marker panels to discriminate mild–moderate from advanced fibrosis [32]. Our study found both Apo A-I and Apo C-III levels to be strongly associated with advanced liver fibrosis; however, when included in the same model, the Apo C-III association remained highly significant while the association of Apo A-I was largely, but not completely, attenuated. This suggests that the two markers may confound each other to some degree, but that there remains an independent effect of each on progressive fibrosis. Nonetheless, inclusion of both Apo A-I and Apo C-III together in the same model did not appreciably improve the predictive accuracy. Therefore, from a clinical standpoint, there is likely to be minimal incremental benefit considering Apo C-III in addition to Apo A-I for assessing advanced stage hepatic fibrosis.

Our research was carried out in a large, tertiary care cohort of CHC patients in the USA, and as such, has several limitations. First is the cross-sectional nature of the study, which prevents us from ascribing a temporal relationship between HCV infection and lipoprotein levels. Second, comparisons between chronic versus cleared infection were not based on matched pairs of subjects before and after treatment. Likewise, another limitation is that we did not have samples from spontaneous clearance cohorts for this study. However, we did control for potential confounding variables and limited our analysis to subjects who were sustained virologic responders to therapy. Finally, lipoproteins were measured in non-fasting samples. While this is not an issue for Apos, levels of triglycerides, in particular, may fluctuate with diet [33]. Nonetheless, at least in cardiovascular disease, non-fasting triglyceride levels are still valuable biomarkers, and may be better predictors of clinical atherosclerotic disease than fasting triglyceride levels [34].

In conclusion, our data indicate an association between low serum Apo C-III levels and CHC infection. Moreover, Apo C-III emerged as the lipoprotein with the strongest independent association with liver fibrosis in multivariable models, yet there may be little incremental value of this marker above and beyond established existing lipoprotein markers of fibrosis progression, such as Apo A-I. Nonetheless, serum Apo C-III levels in chronic HCV infection may provide further clues to the pathogenic mechanism of this virus and deserve further attention as a possible marker of HCV disease progression.

Acknowledgements The authors would like to thank all of the study participants who contributed their biospecimens and data to the Duke Hepatology Clinic Database and Biorepository, and acknowledge Diane Uzarski, Crystal Cates, Chris Delionbach, and Melissa Austin for continued maintenance of this valuable resource. This study was funded in large part by a generous grant from the David H Murdock Institute for Business and Culture via the M.U.R.D.O.C.K. Study and NIH CTSA award 1 UL_1 RR024128-01. Dr. Rowell is supported by NIH Training Grant T32-DK007012-31 at Duke University Medical Center. Dr. Thompson received funding support from the Duke Clinical Research Institute, a generous research gift from the Richard B. Boebel Family Fund, the National Health and Medical Research Council of Australia, and the Gastroenterology Society of Australia. There are no conflicts to disclose.

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