Recognition of Core- and Polymerase-derived immunogenic peptides included in novel therapeutic vaccine by T cells from Chinese chronic hepatitis B patients

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Summary
Chronic hepatitis B (CHB) is one of the major public health challenges in the world. Due to a strong interplay between specific T-cell immunity and elimination of hepatitis B virus (HBV), efforts to develop novel immunotherapeutics are gaining attention. TG1050, a novel immunotherapy, has shown efficacy in an animal study. To support the clinical development of TG1050 in China, specific immunity to the fusion antigens of TG1050 was assessed in Chinese patients. One hundred and thirty subjects were divided into three groups as CHB patients, HBV spontaneous resolvers, and CHB patients with HBsAg loss after antiviral treatment. HBV-specific T-cell responses to pools of HBV Core or Polymerase genotype D peptides included in TG1050 were evaluated. HBV Core- or Polymerase-specific cells were detected in peripheral blood mononuclear cells (PBMCs) from the different cohorts. The frequencies and intensities of HBV Core-specific immune responses were significantly lower in CHB patients than in HBsAg loss subjects. In CHB patients, a dominant pool derived from Polymerase (Pol1) was the most immunogenic. CHB patients with low viral loads (<10^6 IU/mL) were more likely to have a positive response specific to the Core peptide pool. Overall, genotype D-derived peptides included in TG1050 could raise broad and functional T-cell responses in PBMCs from Chinese CHB patients infected with genotype B/C isolates. Core-specific immunogenic domains appeared as “hot spots” with the capacity to differentiate between CHB vs HBsAg loss subjects. These observations support the extended application and associated immune monitoring of TG1050 in China.

KEYWORDS
Core and Polymerase, HBV, HBV genotype, immune response, TG1050

Abbreviations: Ad, adenovirus; ALT, alanine transaminase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; FBS, fetal bovine serum; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NUCs, nucleos(t)ide analogs; PBMCs, peripheral blood mononuclear cells; peg-IFN-α, pegylated interferon α; PMA, phorbol myristate acetate.
Approximately 240 million people are chronically infected by hepatitis B virus (HBV) worldwide, which makes HBV infection a global health burden. An epidemiology study found that the prevalence of hepatitis B surface antigen (HBsAg) for the Chinese population aged 1-59 years was 7.18%. It has been estimated that 20 million patients suffer from CHB infection in China and are at high risk of developing cirrhosis and hepatocellular carcinoma. Current treatments, including nucleos(t)ide analogs (NUCs) and pegylated interferon α (peg-IFN-α), have shown efficacy in inhibiting HBV replication; however, long-term therapy is required and virus elimination is a rare.

CHB patients often display compromised immune responses. Development of broad and robust CD8+ and CD4+ T-cell responses targeting multiple HBV antigens is considered the major contribution to virus control and/or elimination. Several immune therapeutic-based approaches have been developed to overcome impairment of specific immune responses in CHB patients, aiming to induce HBV-specific T-cell responses similar to those found in spontaneous resolvers of the infection. Most HBV immunotherapeutics, the so-called therapeutic vaccines, have been investigated so far in clinical trials have achieved limited success in reaching a functional cure, that is in eliminating circulating HBsAg and provoking anti-HBsAg seroconversion. Sustainability of specifically induced T cells is poor and not associated with a robust antiviral effect in the clinic.

Based on a nonreplicative human adenovirus (Ad) 5 vector, TG1050 is a novel HBV-specific immunotherapeutic, encoding a unique and large fusion protein composed of a modified HBV Core and Polymerase and selected domains of the envelope proteins derived from a genotype D isolate. In a preclinical study, TG1050 induced functional T cells to produce cytokines and displaying cytolytic activity in HBV-persistent mouse models, in which TG1050 had antiviral activities. We have previously shown in a pilot study that T cells from CHB patients infected by nongenotype D isolates can recognize Core-derived epitopes derived from the TG1050-encoded sequence, a bulk of data supportive of the value of the TG1050 in the treatment of large HBV-infected populations.

In this study, we expanded these earlier observations to CHB patients from China. Although vaccination programmes are a priority and quite successful in China, CHB still represents a nationwide priority. Chinese carrier patients are infected mainly by nongenotype D isolates (genotypes B and C). TG1050-similar T101 has been manufactured in China and will enter clinical development in the near future in this country. Our aim was threefold: (i) to analyse the capacity of T cells from CHB Chinese carriers to recognize peptides from two major components of TG1050, the Core and the Polymerase; (ii) in particular, to analyse the association between clinical variables (infecting genotypes, HBsAg levels, status of infection and/or treatment) and capacity to recall effective responses to TG1050-encoded peptides; (iii) to define potential “immunogenic hot spots” within these two antigens to optimize future immune monitoring of TG1050 clinical development.
fetal bovine serum (FBS, Gibco, by Thermofisher scientific, Waltham, Massachusetts, USA) containing 10% DMSO (Sigma, St. Louis, Missouri, USA) and stored in liquid nitrogen. Cryopreserved PBMCs were thawed and rested for 18 hours at 37°C with 5% CO₂ in culture medium including RPMI-1640 (HyClone, USA), with 10% FBS, 1% sodium pyruvate (Gibco, by Thermofisher scientific, Waltham, Massachusetts, USA), 1% HEPES (Gibco, by Thermofisher scientific, Waltham, Massachusetts, USA), 1% antibiotic-antimycotic (Gibco, by Thermofisher scientific, Waltham, Massachusetts, USA) and 1% MEM Non-Essential Amino Acids Solution (Gibco, by Thermofisher scientific, Waltham, Massachusetts, USA). After resting, PBMCs were washed and suspended at 3 x 10⁶/mL in culture medium with 20 ng/mL interleukin (IL)-7 (Peprotech, Rocky Hill, New Jersey, USA) and the 4 peptide pools (average 2.15 μg/mL/peptide) in 48-well plates. Half of the medium was replaced every 2 days with a complete medium supplemented with recombinant IL-2 (50 IU/mL, Peprotech, USA).

2.5 | Enzyme-linked immunospot (ELISPOT) assay

HBV-specific IFN-γ-producing cells were quantified by an ELISPOT assay as described after 10 days of expansion culture.²⁶

Sterile nitrocellulose HA 96-well plates (Millipore Bedford, MA, USA) were coated with 15 μg/mL anti-IFN-γ mAb and incubated overnight at 4°C. The coated wells were filled in triplicate with in vitro stimulated cells (2 x 10⁶/well) with the appropriate HBV peptide pool (average 2.15 μg/mL/peptide) or with medium as a negative control or phorbol myristate acetate (PMA) (12.5 ng/mL)/ionomycin (1 μg/mL) as a positive control.

CTL Analyzer S5 was used to score the number of spots. PBMCs from 6 healthy subjects were also tested with the same peptide pools, and no evident spot was detected, excluding a false positive.

2.6 | IFN-γ intracellular cytokine staining (ICS)

ICS was applied to each ELISPOT-positive sample to determine whether CD8⁺ or CD4⁺ T-cell subsets were responsible for IFN-γ production. Approximately 1 x 10⁶ in vitro-expanded PBMCs were cultured with Core or selected Polymerase peptide pools for 6 hours. PBMCs were stimulated with either medium as a negative control or PMA (12.5 ng/mL)/ionomycin (1 μg/mL) as positive controls. Brefeldin A solution (Biolegend, San Diego, State of California, USA) was added to each well 1 hour after the cells were cultured. After washing, cells were stained with CD3-PE, CD8-FITC and CD4-PercpCy5 and finally fixed and permeabilized to detect intracellular cytokines using anti-IFN-γ APC mAbs. All flow cytometry data were derived from a FACS Calibur flow cytometer (BD, San Jose, State of California, USA) and were analysed with FlowJo 9.6.1 software (Tree Star, Ashland, Oregon, USA). The HBV-specific response was evaluated by subtracting background levels of nonspecific cytokine production observed in medium alone. A response was considered positive if the percentage value of the subset of cells with specific secretion of cytokines was at least the value observed with medium alone and represented at least 0.01% of acquired cells.

2.7 | Statistical analysis

Positive responses of the ELISPOT assay were determined using a statistical method called DFR(eq) (Distribution Free Resampling), proposed by Moodie’s laboratory, 2006.¹⁷ It is based on a permutation resampling method adjusted for multiple comparisons. The aim of this method is to compare the antigen and negative control using permutation resampling.

For continuous variables, a non-parametric Wilcoxon-Mann-Whitney test was performed to compare variable distribution between two groups, and a Kruskal-Wallis test was used for at least three groups. Fisher’s exact test was performed to test the independence between modalities of two categorical variables. Analyses were conducted using SAS® 9.4. Tests were performed at the level of 5%.

3 | RESULTS

3.1 | The demographic and serological characteristics of study subjects

Demographic and serological characteristics of all 130 individuals included in this analysis are summarized in Table 1. Among them, the majority of subjects were male (66.2%) with a median age of 33.5 years. All CHB patients were infected by genotype B or C viruses (32/52). The subjects’ HLA was also screened, and more than half of the subjects were HLA-A2 positive (51.2%, 52.9% and 65.5%, in CHB patients, the spontaneously resolved group and HBsAg loss subjects, respectively). While TG1050 aims at being developed in CHB patients either naïve of treatment (vaccine applied as stand-alone) or under standard of care (vaccine combined with standard of care), in our study, we included three different subject groups: CHB patients, HBV spontaneous resolvers and HBsAg loss subjects. The first group is one of the key target groups of TG1050, and the two other groups provide a priori “positive control groups” more likely to harbour functional HBV-specific T cells. In China, drugs with low antiviral potency or a low genetic barrier are still widely used in CHB patients; distinct treatment pressure may influence host immune responses introducing additional confounding factors in the analysis. Hence, patients currently on antiviral therapy were excluded.

3.2 | IFN-γ-secreting T-cells recognizing TG1050-encoded Core and Polymerase peptides are detected in CHB patients

As HBV-specific responses were hardly detectable ex vivo in samples from CHB patients, short-term T-cell lines were derived from thawed cryopreserved PBMCs as performed by others in the field;¹⁸,¹⁹ cells were stimulated for 10 days with peptide pools covering TG1050-encoded genotype D HBV Core (group of 43 peptides) and Polymerase (group of 206 peptides) sequences.

After 10 days of expansion culture, no evident spot was detected in healthy subjects. We observed that recalls performed with genotype D HBV Core and Polymerase pools of peptides were capable of...
TABLE 1  Demographic and clinical data of subjects

|                         | CHB patients (N = 84) | Spontaneous resolver (N = 17) | HBsAg loss after treatment (N = 29) | P value |
|-------------------------|-----------------------|-------------------------------|-------------------------------------|---------|
| Gender male (%)         | 52 (64.6%)            | 9 (52.9%)                     | 25 (86.2%)                          | .024    |
| Age                     | 33.9 ± 1.10           | 33.6 ± 1.91                   | 40.0 ± 1.99                         | .020    |
| ALT (IU/L)              | 205.3 ± 14.62         | 23.7 ± 2.51                   | 25.7 ± 1.96                         | <.001   |
| AST (IU/L)              | 119.6 ± 9.70          | 23.0 ± 1.79                   | 23.2 ± 1.06                         | <.001   |
| HBsAg (IU/mL)           | 17 617.0 ± 2544.37    | 0.0 ± 0.00                    | 0.0 ± 0.00                          |         |
| HBeAg positive/ negative| 58/26                 | 0/17                          | 1/28                                |         |
| HBV genotype B/C        | 32/52                 | ND                            | ND                                  |         |
| HBV-DNA (IU/mL, log)    | 7.2 ± 0.14            | ND                            | 0.0 ± 0.00                          |         |
| Anti-HBs (IU/mL) (positive/negative) | 5.22 ± 3.14 (4/80) | 426.0 ± 109.97 (15/2) | 116.8 ± 51.12 (19/10) |         |

ND, not determined. All data are represented as the mean ± SEM.

P values were calculated by Fisher’s exact and Kruskal-Wallis tests for comparison of three groups.

stimulating HBV-specific IFN-γ-secreting cells. The Pol1 peptide pool was the most immunogenic with 40.5% responders for the IFN-γ response, followed by Pol2, Core and Pol3 (33.3% vs. 22.6% vs. 15.5%, P = .001, Figure 1A). The Pol1 peptide pool had a more significantly dramatic HBV-specific IFN-γ response, with a mean intensity of 29 spot-forming cells per 10^6 PBMC, than the Core, Pol2 or Pol3 peptide pools (14, 15 and 7 spot-forming cells per 10^6 PBMC, P = .010, Figure 1B). We found that recall of the Pol1 peptide pool induced, on the average, a four-fold increase in the mean values of spot-forming cells compared to the mean values of spot-forming cells in these three groups.

To investigate the breadth of the responses to TG1050-encoded HBV Core and Polymerase peptide pools in CHB patients, we verified the frequencies of the IFN-γ response to one peptide pool, to both Core and Pol peptide pools and to more than one pool stimulation in this population. We observed that the frequencies of responders to more than one pool of restimulation were similar to responders to one peptide pool (23.8% vs. 28.6%, P = .55, Figure 1C).

### 3.3 Comparison of IFN-γ secreting T-cells recognizing TG1050-encoded Core and Polymerase peptides among CHB patients, spontaneous recovery and HBsAg loss subjects

HBV spontaneously resolved subjects and HBsAg loss subjects were also investigated as potential positive references in our study.

In spontaneously resolved subjects and HBsAg loss subjects, the frequencies of IFN-γ-positive response in response to Core, Pol1, Pol2 and Pol3 were 31.3%, 25.0%, 29.4% and 5.9% for spontaneously resolved subjects and 44.8%, 51.7%, 37.9% and 28.6% for HBsAg loss patients (Figure 1A). No significant difference was found among four HBV peptide pools for IFN-γ-positive responses in these two groups. As expected, the frequency of positive IFN-γ responses were significantly higher in HBsAg loss patients compared to CHB patients against the Core peptide pool (44.8% vs. 22.6%, P = .022). Consistent with this finding, the frequency of the IFN-γ response to the Pol1 (51.7% vs. 40.5%), Pol2 (37.9% vs. 33.3%) or Pol3 pool (28.6% vs. 15.5%) was also higher in HBsAg loss patients than in CHB patients, but these differences did not reach statistical significance.

The analysis of IFN-γ ELISPOT intensities revealed a similar general pattern compared to the frequencies of IFN-γ-positive responses in all groups (Figure 1B). The magnitude of the response to HBV Core or Polymerase peptide pools was lower in CHB patients compared with HBV spontaneously resolved subjects and HBsAg loss subjects; the mean values of spot-forming cells were more vigorous in HBsAg loss subjects than in CHB patients after restimulation with the Core peptide pool (24 vs 14 spot-forming cells per 10^6 PBMC, P = .033, Figure 1B). We found that recall of the Pol1 peptide pool induced, though not significantly, a relatively higher number of HBV-specific IFN-γ-producing cells in these three groups.

We also verified the frequencies of the IFN-γ response to one peptide pool, to both Core and Pol peptide pools and to more than one pool stimulation in spontaneous recovery and HBsAg loss groups. Though the frequencies of responders to Core/Pol or to at least more than one peptide pool were lower in CHB patients, no significant difference was detected among the three groups.

### 3.4 Core- or Polymerase- specific T cells are derived from both the CD4+ and CD8+ compartments

Using ICS to analyse IFN-γ production, we further characterized HBV Core or Pol-specific responses in subjects displaying an ELISPOT-positive response. HBV-specific IFN-γ-producing cells could be detected in only a few subjects. FACS analysis confirmed that both CD8+ and CD4+ T cells contribute to the HBV-specific IFN-γ-secreting T-cell responses in samples from all three groups. Figure 2 shows the representative ICS data in CHB patients and HBsAg loss subjects in response to either the Core peptide pool or medium. After HBV peptide pool recall, no significant difference...
was observed between IFN-γ-secreting CD4+ and CD8+ T cells in CHB patients. We noticed that, in HBsAg loss subjects in response to the HBV Pol3 peptide pool, CD4+ T cells produced significantly higher levels of IFN-γ compared to CD8+ T cells (0.22 ± 0.022% vs 0.07 ± 0.033%, P = .034). The frequencies of HBV Core- and Pol3-specific IFN-γ-secreting CD4+ T cells were lower in CHB patients compared to HBsAg loss subjects (0.11 ± 0.148% vs 0.16 ± 0.051%, P = .021; 0.11 ± 0.075% vs 0.22 ± 0.022%, P = .039). Similarly, the frequencies of HBV Core-specific CD8+ T cells were also lower in CHB patients, though they were not significant. The pattern of antiviral T-cell responses may distinguish between CHB and HBsAg loss.

3.5 Impact of HBV viral parameters on recognition of Core- or Polymerase-derived antigens in CHB patients

We analysed whether HBV viral parameters, such as HBV genotypes (B vs C) and viral load, may influence the recognition of TG1050-encoded Core- and Polymerase-derived peptides in Chinese CHB patients.

In our study, HBV genotyping was performed in all samples from CHB patients; thirty-two (38.1%) patients were infected with genotype B virus, while the remaining were infected with genotype C. Among CHB patients, the frequencies of responders (Figure 3A, left panel) and their magnitude of HBV responses (Figure 3A, right panel) were similar between genotype B and C infected patients after in vitro recall of HBV Core or Polymerase peptide pools.

In all CHB patients, HBsAg levels were similar between in vitro IFN-γ ELISPOT responders and nonresponders (Figure 3B, left panel), and the levels did not influence the intensities of HBV-specific IFN-γ responses after in vitro recall (Figure 3B right panel).

Though the viral load levels were not significantly different between in vitro ELISPOT responders and nonresponders (Figure 3C, left panel), the magnitude of response specific to the Core but not the Pol peptide pool was significantly increased in CHB patients with a relative low viral load (<10^6 IU/mL, mean of 33 spot-forming cells per 10^6 PBMC) compared to patients with a high viral load (>10^6 IU/mL, mean of 9 spot-forming cells per 10^6 PBMC) (Figure 3C, right panel). Of note, penalized logistic regression analysis also confirmed that CHB patients with low viral loads less than 10^6 IU/mL were 5.49-fold more likely to have a positive response specific to the Core peptide pool (P = .013).

We also verified whether other factors, such as biochemical and host genetic parameters, may influence recalled HBV-specific immune responses. No impact of HBeAg status (Figure 4A), ALT (Figure 4B) or AST on the frequencies of HBV-reactive responses was observed in CHB patients. However, the intensity of HBV-specific IFN-γ responses after HBV Pol2 peptide pool recall was significantly higher in CHB patients displaying more than 2N ALT levels, compared with low ALT patients (Figure 4C). Distinct antiviral treatments, such as NUCs, IFN alpha or combination treatment, do not influence the frequencies of reactive HBV responses.

4 DISCUSSION

HBV chronic infection can be controlled but is rarely cured with first-line treatment regimens. Restoration of host antiviral immunity against HBV is considered an important strategy in the currently developed novel therapeutic approaches. Recently, a novel active targeted immunotherapeutic (therapeutic vaccine) TG1050 has been developed (Phase I clinical trial, NCT02428400). In preclinical study, TG1050 displayed a number of immunological features similar to those found in HBV spontaneous resolvers together with the capacity to exert antiviral activity in an HBV-persistent murine model.
Here, we focused on the analysis of immune responses targeting the two major antigens encoded by TG1050, Core and Polymerase from a genotype D sequence and extended our study to a Chinese CHB population who could benefit from treatment by TG1050. We demonstrated that TG1050-encoded genotype D-derived Core and Polymerase peptides were capable of stimulating functional HBV-specific IFN-γ-secreting CD4⁺ and CD8⁺ T-cell responses. Furthermore, within these two antigens, we defined Core-reactive immune responses targeting apparent “immunogenic hot spots.” These peptide hot spot sequences may provide different immune signatures distinguishing between CHB patients and HBsAg loss subjects. Further studies are required to analyse their value and potential use in future immune-monitoring companion assays to be implemented in clinical developments. Finally, our study suggested that viral load...
HUANG et al. may influence the capacity to recall effective responses to Core peptides, while viral genotype and HBsAg do not impact HBV Core- or Polymerase-reactive responses. In CHB patients, HBV-specific effector T cells are functionally exhausted by continued antigen exposure and multiple regulatory pathways. Circulating HBV-specific T cells are therefore difficult to detect ex vivo. The aim of immunotherapeutic vaccine is to reactivate functional HBV-specific T cells. Here, we demonstrated that peptides derived from TG1050-encoded genotype D HBV sequences based on Core and Polymerase are capable of reactivating functional HBV-specific IFN-γ secreting T-cell responses in CHB patients and in resolved subjects. The Polymerase peptide pool, especially Pol1, was more immunogenic, with high frequency and intensity responses in CHB patients. As reported by Rehermann’s team, HBV Polymerase-specific immune responses were less frequent, though not significantly, in CHB patients compared to HBsAg loss subjects. These Pol responses appear lost in spontaneous resolvers.

Compared with the Pol1 peptide pool, Core pool was less immunogenic in CHB patients. Both frequencies and magnitudes of response were significantly lower in these patients compared to HBsAg loss subjects. We also showed that both CD4+ and CD8+ T cells contributed to the anti-HBV immune responses by secreting IFN-γ. CD4+ T cells are robust producers of cytokines and required for the efficient development of effector CD8+ CTLs. Our IFN-γ ICS analysis revealed that especially HBV Core IFN-γ-secreting CD4+ subsets were significantly lower in CHB patients compared with HBsAg loss subjects. Collectively, the pattern of antiviral HBV Core-specific T-cell responses may distinguish the immune profile between CHB patients and HBsAg loss subjects. Our results suggest an important immune signature related to CHB clinical control seroconversion and may support the development of specific assays for future immune monitoring of TG1050 in the clinic.

We did not analyse TG1050-encoded HBV envelope-reactive immune responses due to the limitation of blood collection. In addition, HBV envelope response is less immunogenic compared to Core/Polymerase domain antigens. In patients displaying HBsAg seroclearance following antiviral therapy, a low to undetectable frequency of CD4+ and CD8+ T-cell immune response to envelope antigens was detected in Chinese subjects. Therapeutic vaccines composed of the unique envelope antigen have so far failed or shown very limited effects in the clinic.

**FIGURE 3** HBV-specific T-cell response and HBV virological parameters (genotype, HBsAg and HBV-DNA) in CHB patients. Comparison of A, percentages of in vitro IFN-γ ELISPOT responders and magnitudes (mean ± SEM) of responses in genotype B- or C-infected subgroups; B, HBsAg levels in IFN-γ ELISPOT responder and nonresponder subgroups and magnitudes of responses in CHB patients with low (≤1000 IU/mL) and high (>1000 IU/mL) HBsAg levels. C, HBV viral load levels in IFN-γ ELISPOT responder and nonresponder subgroups and magnitudes of responses in CHB patients with low viral load (<10^5 IU/mL) and high viral load (>10^6 IU/mL) subgroups. The differences were evaluated by Fisher’s exact test and Wilcoxon-Mann-Whitney test.
We also address the question of whether both virus and host factors may influence the detection of broad T-cell immune responses. Certain early studies have indicated that HBV-specific T-cell quantity is determined by the virological and clinical profiles of the patients, without any influence of viral diversity.

We also verified whether other viral and biochemical parameters, such as HBsAg level and viral load, impact CHB patient host immune responses. HBsAg level represents a very useful marker in the clinical management of chronic HBV, able to predict the response to antiviral therapy and to help in optimizing the clinical classification of patients. Variable HBsAg levels have been postulated to reflect different degrees of immune control. In a small cohort study, the breadth of the anti-Core-specific T-cell response was inversely correlated with serum HBsAg concentrations and HBV-DNA and ALT levels in untreated HBeAg-negative CHB patients. In our study cohort including both HBeAg+ and HBeAg− CHB patients, the intensity of HBV Core- but not Polymerase-specific T cells was increased but did not reach a significant level in CHB patients displaying low HBsAg levels.

Until today, a definitive CHB cure is still a challenge. Viral clearance and long-lasting immune protection are the two major conditions in the modern concept of a functional cure. In combination with small molecules, therapeutic vaccines may be able to reverse the dysfunctional immune state of CHB and reach an HBV cure. TG1050’s preclinical study previously demonstrated both efficacy of viral reduction and functional immune response restoration in murine models. Sequential combination therapy with NUCs and multi-injection of TG1050 might have better CHB treatment outcomes in the future. The reinforced description provided here that TG1050-encoding HBV genotype D-derived peptides can raise a broad and functional T-cell response in PBMCs from genotype B- or C-infected Chinese patients supports the clinical development of this novel immunotherapeutic in China.

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DISCLOSURES

The authors have no commercial or other association that might pose a conflict of interest.

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