New Microbiologica, 45, 3, 193-198, 2022, ISSN 1121-7138

FULL PAPER

Lasting Effects of *Helicobacter pylori* Infection on the Microbial Communities of Patients with and without Small Intestinal Bacterial Overgrowth

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SUMMARY

Gastrointestinal (GI) microbial populations are important in maintaining normal functioning of the GI by preventing disorders. Dysbiotic microbiota may increase the likelihood of small intestinal bacterial overgrowth (SIBO), a syndrome associated with significant morbidity. We aimed to investigate the microbiota populations of patients with SIBO.

Patients with symptoms of SIBO were consecutively enrolled; they underwent a SIBO hydrogen breath test and stool was collected for microbiome analysis by sequencing of the 16S rRNA.

Of the 55 patients recruited, 42 (76.4%) were positive for SIBO. When visualizing the bacterial β-diversity, a sub-cluster of patients was identified. Further examination of these patients' records revealed previous treatment for *Helicobacter pylori* (*HP*). Microbiome analysis of these patients demonstrated a significant decrease in β-diversity (p-value<0.001) compared to patients without previous *HP* therapy. Furthermore, β-diversity was significantly different in this subgroup, and several bacterial taxa were differentially expressed, including one from the genus *Methanobrevibacter*, which was reduced in patients that previously underwent *HP* treatment.

Our findings suggest that while symptoms associated with SIBO may cause dysbiosis, there was no differentiation in fecal microbiome composition based on SIBO diagnosis. Furthermore, our results support previous observations regarding antibiotic-altered microbiota with effects extending two and three years post-treatment.

Received March 24, 2022 *Accepted* June 13, 2022

INTRODUCTION

Small intestinal bacterial overgrowth (SIBO) is defined as an abnormal increase in bacterial count in duodenal aspirate. The clinical presentation varies from mild, nonspecific symptoms such as abdominal pain, flatulence, and bloating to a full-blown malabsorption syndrome with diarrhea and weight loss (Bures *et al.*, 2010). A similar set of symptoms can also be associated with a range of other gastrointestinal (GI) conditions, including irritable bowel syndrome (IBS) (Major *et al.*, 2014; Raskov *et al.*, 2016), celiac disease (Al-Toma *et al.*, 2019) and carbohydrate malabsorption (Hammer *et al.*, 2012; Montalto *et al.*, 2013). A diagnosis of SIBO is typically made when 10^5 or more colony-forming units per milliliter of duodenal aspirate are measured (Noa *et al.*, 2020), but the actual prevalence of SIBO is unclear because many cases go undiagnosed. Rates are higher in older individuals, reaching an estimated 50% in the over-75-year age group, owing to age-related disturbances in gastric acid production, intestinal motility and other natural defense mechanisms. Patients with an underlying gastrointestinal pathology, such as chronic pancreatitis, immune system dysfunction, functional dyspepsia, gastroparesis, celiac disease, and inflammatory bowel disease (IBD), are also more susceptible to SIBO (Shah *et al.*, 2019).

Risk factors associated with SIBO include: structural or anatomic abnormalities such as small intestine diverticula, motility disorders such as gastroparesis or chronic intestinal pseudo-obstruction, IBS and metabolic disorders such as diabetes (Achufusi *et al.*, 2020; Ghoshal *et al.*, 2020; Castiglione *et al.*, 2000).

Key words:
Dysbiotic microbiota, microbiome analysis, antibiotic-altered microbiota.

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Further, the gut microbiome consists of bacteria, archaea, fungi and viruses with a specific balance which, if disturbed, may lead to GI dysfunction including SIBO (Achufusi *et al.*, 2020; Ghoshal *et al.*, 2020). Previous studies that examined microbiota dysbiosis in SIBO patients typically focused on local small intestine microbiota composition (Saffouri *et al.*, 2019; Leite *et al.*, 2020) and reduced diversity as well as on a number of differentially abundant bacteria that were observed among SIBO patients compared to healthy controls (Saffouri *et al.*, 2019). When comparing patients with GI symptoms but no SIBO diagnosis to those with a diagnosis, one study found significant differences in alpha and beta diversity between the groups (Leite *et al.*, 2020), while another did not (Saffouri *et al.*, 2019). Differences were also found among SIBO-positive patients with and without symptoms (Saffouri *et al.*, 2019).

A lactulose hydrogen breath test (LaBT) is an important means of clinical diagnosis of SIBO. It is simple, non-invasive and requires only a short period of fasting (Perets *et al.*, 2017). SIBO is typically treated with antibiotics, namely neomycin, ciprofloxacin, and especially rifaximin (Perets *et al.*, 2017). This regimen, while effective in 16.7-100% of cases [e.g., for rifaximin (Kim *et al.*, 2017)], may also cause long-term gut dysbiosis of another kind, as demonstrated previously for a range of antibiotic regimens (Kim *et al.*, 2017). Treatment of SIBO generally lasts 7 to 14 days (Bae *et al.*, 2015), although a recent study suggested that a longer period of up to 12 weeks is needed for complete eradication, corresponding to the time to normalization of repeated LaBTs (Bae *et al.*, 2015).

In this preliminary descriptive study, we examined the gut microbiome from fecal samples collected from patients suffering from similar GI symptoms without any other diagnosis of GI disease in order to determine if there is a relationship between the microbiome and a SIBO diagnosis. We did not observe significant differences in gut microbiota of patients suffering from GI symptoms regardless of SIBO diagnosis; however, we did find lasting effects of *Helicobacter pylori* treatment (*HP*) on the microbiota of a subset of patients. This unique finding is in line with other studies that confirmed an enormous impact on human gut microbiota even in short-term antibiotic treatments (Jernberg *et al.*, 2007; Buffie *et al.*, 2012).

MATERIALS AND METHODS

Study population

Patients were referred by a consultant gastroenterologist to the gastroenterology laboratory at Rabin Medical Center (RMC) for LaBT due to suspected SIBO and as a part of their IBS assessment. Inclusion and exclusion criteria were as follows: 55 Patients (29.1% male, age 25.2±4.0 years) with intestinal symptoms such as diarrhea, nausea, bloating, flatulence, abdominal pain and constipation were recruited. Patients who had consumed antibiotics in the 6 months previous to enrolment were excluded. Patients with previous ROME III criteria for IBS or other organic GI disease that might explain GI symptoms mentioned above (celiac, Crohn's, ulcerative colitis, etc.) were also excluded. Following recruitment, all patients underwent LaBT for SIBO diagnosis. In addition, patients were asked to provide a stool sample (55 samples in total). All samples were collected and analyzed prior to SIBO treatment.

SIBO lactulose hydrogen breath test

Lactulose hydrogen breath test was performed to evaluate SIBO according to standard protocols (Perets *et al.*, 2017). In short, each patient was asked to consume a low carbohydrate diet 24 hours prior to the test in order to minimize basic hydrogen discharge, fast for 12 hours and perform a chlorhexidine mouth wash on the morning of the test. Smoking and physical activity were prohibited in the morning before and during the test. After measuring baseline hydrogen, patients were given 15 g of lactulose dissolved in 50 ml of water. Samples of end-expiratory air were collected after baseline measurement every 15 minutes and up to 90 minutes. The test results were considered positive when hydrogen concentration exceeded 20 PPM above baseline.

Microbiome analysis

DNA was extracted from all collected samples using the PureLink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher; Waltham MA, USA) according to the manufacturer's instructions and following a 2 min bead beating step. Then the variable V4 region was PCR-amplified using the 515F and 806R barcoded primers following the Earth Microbiome Project protocol (Caporaso *et al.*, 2012). Each PCR reaction consisted of 25 µL PrimeSTAR Max PCR mix (Takara Kusatsu, Shiga, Japan), 2 µM of each primer, 17 µL of ultra-pure water, and 4 µL DNA template. Thermal cycler conditions were as follows: 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 5 sec, and extension at 72°C for 20 sec, followed by a final elongation at 72°C for 1 min. Amplicons were purified using AMPure magnetic beads (Beckman Coulter; Indianapolis, IN, USA) and quantified using the Picogreen dsDNA quantitation kit (Invitrogen, Thermo Fisher; Waltham, MA, USA). Equimolar amounts of DNA from individual samples were pooled and sequenced using the Illumina MiSeq platform at the Genomic Center at the Bar-Ilan University Azrieli Faculty of Medicine in Tzfat, Israel. Appropriate negative and positive controls were included.

The 16s rRNA gene sequence data set was processed with QIIME2 version 2020.8 (Bolyen *et al.*, 2019) using default parameters. DADA2 (Callahan *et al.*, 2016)

was utilized to filter out noisy sequences, correct errors in marginal sequences, remove chimeric sequences, remove singletons, and de-replicate sequences into amplicon sequence variants (ASVs) with 100% sequence similarity based on the Greengenes closed reference database (DeSanyis *et al.*, 2006). The resulting ASV feature table was exported to R v. 4.0.4. Mitochondrial and chloroplast DNA and undefined phyla were filtered out. Samples were rarefied to 19,053 reads per sample (1 sample lost), and the rarefied data was used for downstream analysis using the *phyloseq* package (McMurdie *et al.*, 2013). The α-diversity of the rarefied microbiome samples was computed using Faith's phylogenetic distance metric (Faith's PD) and compared between groups using Welch's ANOVA (SIBO vs. non-SIBO) or ANOVA for unbalanced design (*HP* vs non-*HP*). Community-wide divergence (β-diversity) was assessed using multivariate analysis based on community dissimilarity. We used weighted and unweighted UniFrac measures to assess dissimilarity (Lozupone *et al.*, 2005). Both measures consider evolutionary distance between ASVs; weighted UniFrac is affected by both the composition and abundance of ASVs, whereas unweighted UniFrac is based only on ASV presence or absence. Multi-dimensional scaling (MDS) analyses were performed on UniFrac distances. We first compared within-group dispersion (*vegan* R package) (Oksanen *et al.*, 2020). We then performed distance-based per mutational multivariate analysis of variance (PERMANOVA, *vegan* R package). Lastly, we examined differential abundance of genera between groups using the R package *DESeq2* (Love *et al.*, 2014) within *phyloseq* to identify genera significantly more abundant in either of the two groups.

Ethics Approval

This study was approved by the RMC Institutional Review Board. Informed consent was obtained from all participants in accordance with Helsinki ethics permit # 0036-15-RMC from the RMC Institutional Review Board.

RESULTS

Of the 55 recruited patients, 42 had a positive SIBO diagnosis (76.4%). There was no difference in diagnosis rate for male (18.8%, n=16) and female (34.5%, n=39) patients (Fisher exact test, p=0.734). When examining diagnosis-related changes in the gut microbiome, we did not find differences between symptomatic patients with and without a positive diagnosis (*Figure 1a, b*). We did, however, observe a sub-cluster in the principal coordinate space when examining the β-diversity of the cohort (*Figure 1b*). Upon re-examination of our data, we found significant differences in SIBO diagnosis between those previously infected with *HP* and those who were not (Fisher exact test, p-value=0.006; *Table 1*), with higher incidence among SIBO-negative patients. We also noticed pronounced decreases in α-diversity of patients previously infected with *HP* (ANOVA, p-value<0.001;

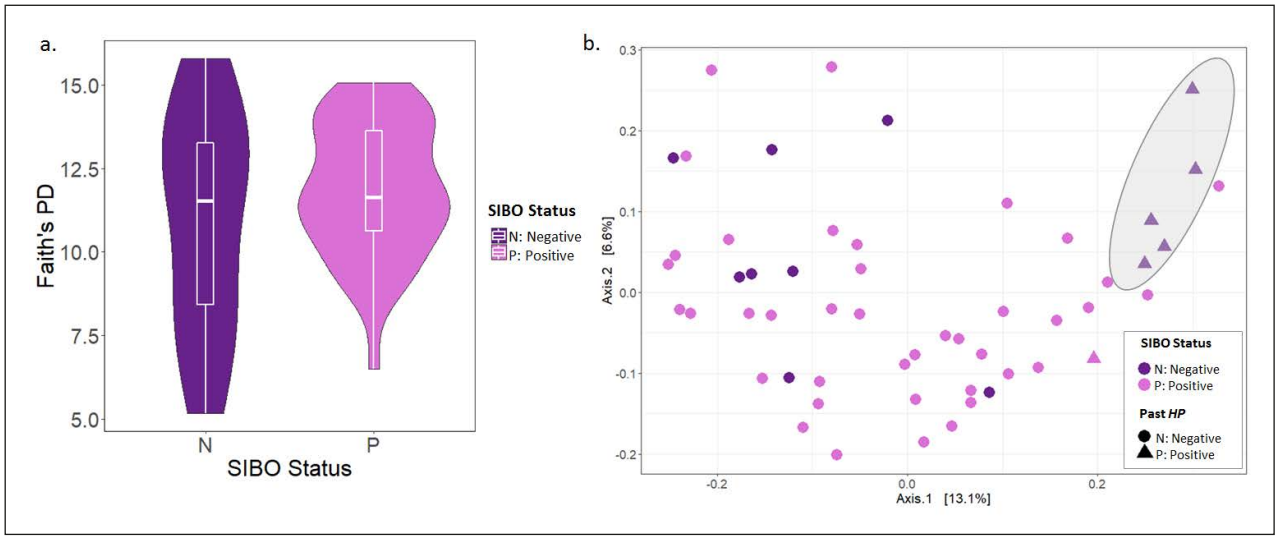


Figure 1 - *No differences were observed in fecal microbiota composition of symptomatic patients with and without SIBO. We found no differences in (a) α-diversity using Faith's PD (Welch's ANOVA, p-value=0.353) or (b) β-diversity based on unweighted UniFrac, (PERMANOVA p-value=0.125). We did, however, identify a subcluster in the principal coordinate space, denoted by the gray ellipse. Dark purple represents patients with a negative SIBO diagnosis and light purple denotes a positive diagnosis. Following retrospective examination of patient medical records, we discovered that the subcluster represents patients who were previously affected by Helicobacter pylori (HP; triangles).*

Table 1 - *Co-occurrence of SIBO and previous Helicobacter pylori (HP) infection*[a].

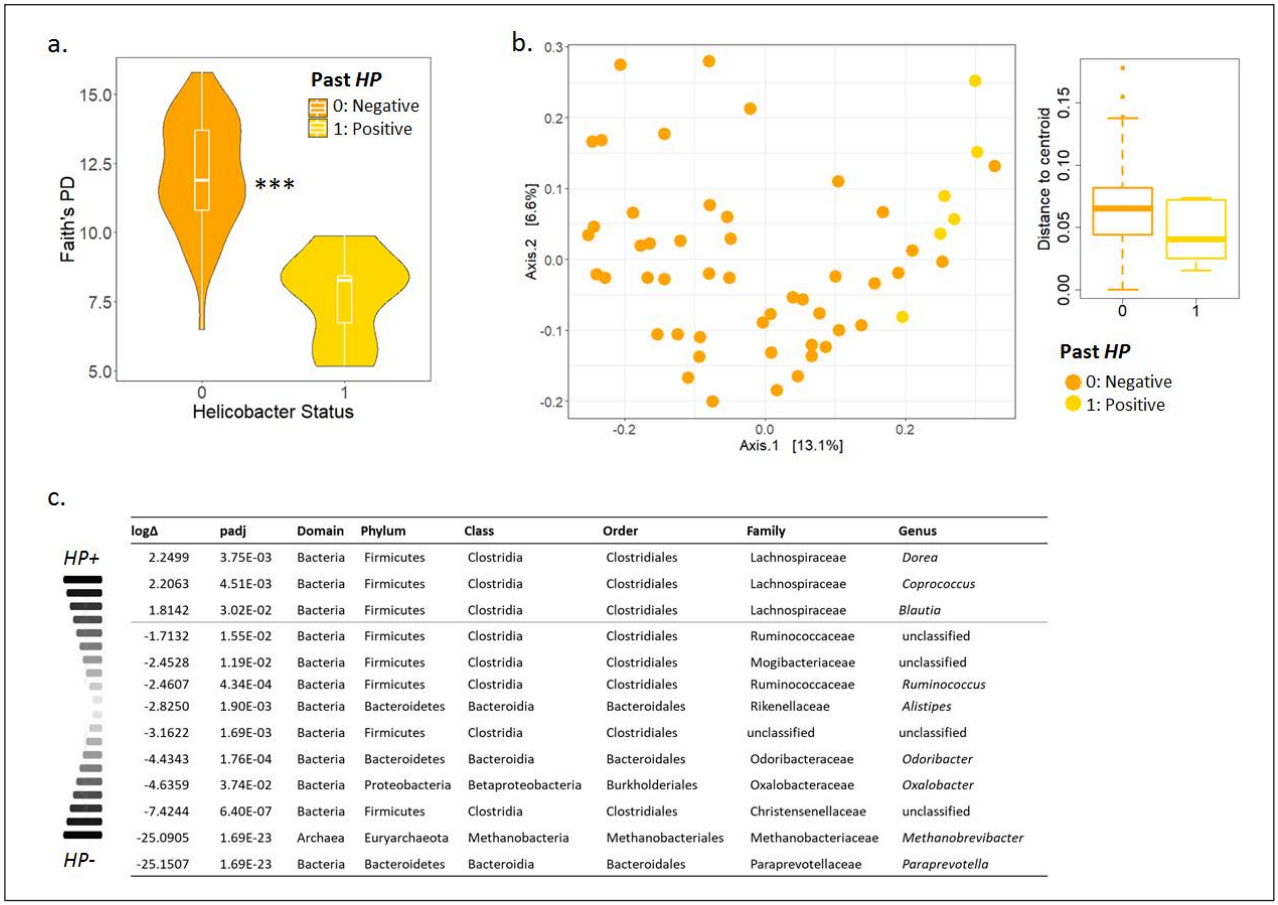
	HP	*No HP*
SIBO-Positive	2	40
SIBO-Negative	5	8

[a]There is non-independence in the co-occurrence pattern, such that a greater proportion of SIBO-negative patients had *HP* in the past than those found to be SIBO-positive (Fisher exact test, p-value=0.006).

Figure 2a). Further, bacterial composition between the groups (β-diversity) was significantly different (weighted UniFrac, PERMANOVA, p-value=0.020; unweighted UniFrac, PERMANOVA, p-value<0.001; *Figure 2b*), and there was a trend toward increased dispersion from the centroid in the group of patients not previously infected with *HP* when using the weighted UniFrac measure (betadisper, p-value=0.093; *Figure 2b*). Using DESeq2, we identified 13 differentially abundant taxa between the groups (p-adjusted<0.05, *Figure 2c*). Of note was the genus *Methanobrevibacter*, which had higher relative abundance in patients not previously diagnosed with *HP*.

DISCUSSION

In this study, we examined how a diagnosis of SIBO affects fecal microbiome composition compared with GI-symptomatic patients without a SIBO diagnosis. Since our cohort was relatively homogenous in terms of patients with similar GI symptoms and no known GI disease, we refrained from comparing them to healthy individuals; our main focus was microbiota differences within a symptomatic group. In a previous study, it was demonstrated that a cutoff of >10^3



	logΔ	padj	Domain	Phylum	Class	Order	Family	Genus
HP+	2.2499	3.75E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Dorea*
	2.2063	4.51E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Coprococcus*
	1.8142	3.02E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Blautia*
	-1.7132	1.55E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
	-2.4528	1.19E-02	Bacteria	Firmicutes	Clostridia	Clostridiales	Mogibacteriaceae	unclassified
	-2.4607	4.34E-04	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*
	-2.8250	1.90E-03	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*
	-3.1622	1.69E-03	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified	unclassified
	-4.4343	1.76E-04	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	*Odoribacter*
	-4.6359	3.74E-02	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Oxalobacter*
	-7.4244	6.40E-07	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	unclassified
	-25.0905	1.69E-23	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobrevibacter*
HP-	-25.1507	1.69E-23	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Paraprevotellaceae	*Paraprevotella*

Figure 2 - *Significant differences were found in fecal microbiota composition of symptomatic patients who were and were not previously diagnosed with Helicobacter pylori (HP) infection. (a) We found significantly reduced α-diversity among those previously treated for HP using Faith's PD (ANOVA, p-value<0.001). (b) β-diversity based on weighted (not shown) and unweighted UniFrac was also significantly different (PERMANOVA p-value<0.001). While dispersion from the centroid was similar when using the unweighted UniFrac measure, there was a tread toward decreased dispersion in the HP group when using the weighted measure (p=0.093). We identified 13 differentially expressed taxa with an FDR-adjusted p-value (padj)<0.05. Those with a positive log-2-fold-change (logΔ) had higher relative abundance in the HP group. Orange represents patients without previous HP and yellow denotes those with a previous HP infection.*

CFU/mL for the definition of SIBO is important in recognizing a unique Proteobacterial profile (Leite *et al*., 2020). The researchers also found a positive correlation between the microbiome-associated findings and the clinical manifestation of SIBO. In contrast with this study (Leite *et al*., 2020), we did not find differences in microbial composition among our study groups. This could stem from differences in SIBO diagnosis criteria. Our results are in line with Saffouri *et al.* (2019), who found significant differences in intestinal dysbiosis when comparing GI-symptomatic patients to healthy controls but no differences when comparing symptomatic patients with and without SIBO. Thus, similar bacteria are likely responsible for GI symptoms on the whole, rather than being specifically associated with SIBO diagnosis. Further, the disagreement with Leite *et al.* (2020) could be explained by the fact that their microbiome analysis was performed on liquid samples obtained from the duodenum during upper GI endoscopy, while our samples were collected during defecation and represent the entire GI environment.

Of note, we identified a subcluster of patients when examining microbial β-diversity. This cluster was composed of patients previously infected with and treated for *HP*. These patients were all prescribed a first-line triple therapy treatment of clarithromycin, amoxicillin and a proton pump inhibitor (Malfertheiner *et al*., 2017) 24-36 months prior to study recruitment. The finding of significantly reduced α-diversity among patients previously treated for *HP* is noteworthy because it suggests that the effects of intensive *HP* antibiotic treatment are long-lasting. Not only was richness reduced among these patients, but their community composition (β-diversity) was also different from patients not previously infected with *HP*, both in taxa identity (unweighted UniFrac) and taxa relative abundance (weighted UniFrac). The dispersion of these patients was also slightly reduced, though this may be an artifact due to small sample size and unbalanced design. We identified 13 genera driving the community differences between patients with and without previous *HP* diagnosis. Importantly, the genus *Methanobrevibacter* was less abundant among patients previously diagnosed with *HP.* This methane-producing genus is known to have therapeutic effects (Chaudhary *et al*., 2018). Thus, its absence could be the source of GI symptoms in those patients who were previously infected by *HP* and less likely to be diagnosed with SIBO.

Our study suggests a stable profile across GI-symptomatic patients regardless of SIBO status. Further, we uncovered long-term, lasting effects of first-line treatment of *HP* infection three years post-treatment, which is in concordance with previous studies that reported a tremendous impact on GI microbiota, including diversity decreases after just one week or less of antibiotic administration (Jernberg *et al*., 2007) and a single dose of Clindamycin for *Clostridium difficile* infection (Buffie *et al*., 2012). Since all patients were symptomatic, it would be interesting to examine metabolite profiles; functional differences between those with and without previous *HP* infection may be less pronounced than observed microbial differences.

Acknowledgments

We thank Prof. Omry Koren (Microbiome Research Lab, Azrieli Faculty of Medicine, Bar-Ilan University, Zefat, Israel), who served as a scientific advisor and critically reviewed the study proposal.

The authors have no conflicts of interest to disclose. No funding was received for the preparation of this manuscript.

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