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FULL PAPER

Impact of oral antiviral therapy against HCV on gut microbiota. A prospective study

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SUMMARY

The intestinal microbiota plays a fundamental role in physiological homeostasis as well as in pathologic conditions. Hepatitis C virus is the leading cause of chronic liver diseases worldwide. The treatment of this infection has been revolutionized by the availability of direct-acting antiviral agents which guarantee a high rate (about 95%) of viral clearance. Few studies have assessed the change in the gut microbiota of patients treated with direct-acting antiviral agents against HCV, and many aspects still need to be clarified. The aim of the study was to evaluate the effects of antiviral therapy on gut microbiota.

We enrolled patients with HCV-related chronic liver disease attending the Infectious Diseases Unit of the A.O.U. Federico II of Naples from January 2017 to March 2018 and treated with DAAs. For each patient, a fecal sample was collected and analyzed for the assessment of microbial diversity before the start of therapy and by SVR12 time. We excluded patients who had received antibiotics in the previous 6 months.

Twelve patients were enrolled (6 male, 8 genotype 1 (1 subtype 1a), 4 genotype 2). Fibrosis scores were F0 in 1 patient, F2 in 1 patient, F3 in 4 patients and cirrhosis in the remaining 6 (all in Child-Pugh class A). All were treated with DAAs for 12 weeks (5 with Paritaprevir-Ombitasvir-Ritonavir-Dasabuvir, 3 with Sofosbuvir-Ledipasvir, 1 with Sofosbuvir-Ribavirin, 1 with Sofosbuvir-Daclatasvir, 1 with Sofosbuvir-Velpatasvir) and 100% achieved SVR12. In all patients, we observed a trend in reduction of potentially pathogenic microorganisms (i.e., Enterobacteriaceae). Furthermore, a trend of increase in α-diversity was observed in patients by SVR12 compared to baseline. This trend was markedly more evident in patients without liver cirrhosis than in those with cirrhosis.

Our study shows that viral eradication obtained with DAA is associated with a trend in restoring the heterogeneity of α-diversity and in reducing the percentage of potentially pathogenic microbial species, although this benefit is less evident in patients with cirrhosis. Further studies with larger sample size are needed to confirm these data.

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INTRODUCTION

The intestinal microbiota has been the focus of attention of the international scientific community for the last fifteen years; therefore, more than 12,900 studies have been produced on this topic. The intestinal microbiota can be involved in the development of numerous pathologies, such as diabetes and obesity, but also in neurodegenerative, rheumatological and oncological pathologies (Cani *et al*., 2018; De Filippis *et al*., 2018), as well as in neuropsychiatric disorders such as anxiety, depression and autism (Scher *et al*., 2011; Foster *et al*., 2012). According to several studies, gut microbiota dysbiosis is also found in patients with liver diseases (Abenavoli *et al*., 2013; Scarpellini *et al*., 2015, a). Indeed, clinical data show significant differences in the composition of bacterial microbiota in several liver diseases such as NAFLD (Non-Alcoholic Fatty Liver Disease), ALD (Alcoholic Liver Disease) and viral hepatitis compared with healthy subjects (Haque *et al*., 2016; Scarpellini *et al*., 2016, b). Recent studies have revealed differences between the microbiota of patients with chronic liver disease without cirrhosis compared to those with liver cirrhosis (Gentile *et al*., 2019, a). However, compared to what is ob-

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served in NAFLD and ALD, changes in intestinal microflora in subjects with hepatitis C are completely different (Gentile *et al*., 2019, a). Hepatitis C virus infection is the leading cause of chronic liver disease throughout the world: it is estimated that around 71 million people are affected. Italy is a country with a high prevalence of HCV infection (Buonomo et al., 2018; Fukui *et al*., 2015). Some studies, in particular the study conducted by Bajaj et al. and the study conducted by Aly *et al*., showed that in patients with HCV infection the composition of the microbiota is altered in a peculiar way, with a predominance of Bacteroidetes, Prevotella and Acinetobacter, Veillonella, Phascolarctobacterium and Faecalibacterium. On the other hand, healthy controls show higher levels of Ruminococcus, Clostridium, and Bifidobacterium (Bajaj *et al*., 2016; Aly *et al*., 2016, Munteanu *et al*., 2014; Gentile *et al*., 2019, b). The recent advent of new therapies in the treatment of HCV infection has represented a real revolution. These drugs, named DAAs (Direct-Acting Antivirals) are characterized by the achievement of a cure rate in 95% of cases (Scotto *et al*., 2019; Di Minno *et al*., 2020; Chuaypen *et al*., 2021). Few studies have been conducted so far on the change in the gut microbiome after viral clearance achieved by means of DAAs in HCV infection. In particular, a study conducted by Chuaypen N. *et al.* highlighted a short-term effect of DAAs in restoration of microbial dysbiosis with favorable changes in the gut microbiota after viral eradication (Wellhöner *et al*., 2021). Another study by Wellhöner *et al.* highlighted that the achievement of SVR 24/48 in patients with chronic HCV was associated with changes in the intestinal microbiota, but these changes were observed only in patients without cirrhosis (Dietrich *et al*., 2017).

In the present study, we aimed to assess intestinal microbiota changes in patients with HCV-related chronic liver disease before vs. after therapy with direct-acting antiviral agents.

MATERIALS AND METHODS

We enrolled patients with HCV-related chronic liver disease attending the Infectious Diseases Unit of the A.O.U. Federico II of Naples, from January 2017 to March 2018 and treated with DAAs. Fibrosis was evaluated by the mean of Fibroscan®. The severity of liver disease was graduated according to the degree of liver fibrosis by Metavir stage or clinical signs. The Metavir score was estimated with a FibroScan© exam performed within 6 months before the beginning of the antiviral treatment. Moreover, clinical cirrhosis was identified according to the presence of clinical, biochemical and ultrasound signs, including a blood platelet count below 100,000/mm^3, hypertrophy of the caudate lobe, nodularity of liver surfaces, altered straightness of hepatic veins, ascites, portosystemic encephalopathy, esophageal varices, and ultrasound evidence characterizing liver cirrhosis. Decompensated cirrhosis was defined as a cirrhosis in Child-Pugh stage of at least B7, while advanced liver fibrosis was defined as the presence of Metavir score ≥F4 or clinical cirrhosis (Canani *et al*., 2017).

For each patient, a fecal sample was collected within 1-2 weeks before starting therapy and by SVR12 time. Subjects with the following inclusion and exclusion criteria were enrolled:

- Inclusion criteria: positivity for HCV-RNA; HBsAg negativity; anti-HIV negativity; age >18 years; intention to participate in the study and signature of informed consent.
- Exclusion criteria: age <18 years; coinfection with HBV or HIV; inability to understand or sign informed consent; any other condition that in the opinion of the experienced person could make the patient unsuitable for enrollment or that could interfere with the patient's participation in the study and its completion; use of any antibiotic, antifungal or probiotic in the past 90 days; BMI (Body Mass Index) >30.

The enrolled subjects were equipped with a special kit for the collection of fecal samples, together with the instructions for sampling. These samples were stored at -80° C until the time of analysis. Collection, transport, and storage were carried out following the Standard Operating Procedures (SOP) defined by the International Human Microbiome Standard Consortium (IHMC SOP 04).

Microbial DNA extraction, 16S rRNA gene sequencing and data analysis

Fecal microbial DNA was extracted using the procedures outlined by the IHMC SOP 07 at the Department of Agricultural Sciences of the University of Naples Federico II. Fecal microbiota was analyzed by sequencing the V3-V4 regions of the 16S ribosomal RNA gene, with a procedure and primers previously described (Magoč *et al*., 2011; Besaury *et al*., 2017). Amplicons were purified, indexed, and sequenced on a MiSeq Illumina platform according to the Illumina 16S metagenomic sequencing library preparation protocol, yielding 2x250-bp, paired-end reads.

Demultiplexed, forward, and reverse reads were joined by using FLASH (Buvè *et al*., 2014). Joined reads were quality trimmed (Phred score >20) and short reads (<250 bp) were discarded by using Prinseq (Lanan *et al*., 2016).

High-quality reads were then imported into QIIME 1.9 (Lanan *et al*., 2016). Operational taxonomic units (OTU) were picked at 97% of similarity using a de novo approach and the uclust method, and taxonomic assignment was obtained by using the RDP classifier and the Greengenes database (Besaury *et al*., 2017), following a pipeline previously reported (Besaury *et al*., 2017). In order to avoid biases due to the different sequencing depth, OTU tables were rarefied

Table 1 - *Demographic and clinical characteristics of the subjects enrolled (n=12).*

Age (median, IQR)	71.38 (54.23-75.79)
Gender	
M	6 (50%)
F	6 (50%)
HCV-RNA (IU/ml; median, IQR)	950,000 (34,000-2,100,000)
Genotype	
1a	1 (8.3%)
1b	7 (58.3%)
2	4 (33.3%)
Albumin (g/dl; median, IQR)	3.7 (2.9-4.5)
Platelets (elements/μL; median, IQR)	168,000 (92.000-383.000)
INR (median, IQR)	1.1 (0.9-1.4)
ALT (IU/l, median, IQR)	54 (33-124)
AST (IU/l, median, IQR)	49 (28- 96)
Tot. Bil. (mg/dl; median, IQR)	0.9 (0.7-1.5)
Staging (Metavir score)	
F0	1 (8.3%)
F1	0 (0)
F2	1 (8.3%)
F3	4 (33.3%)
F4	6 (50%)
Clinical cirrhosis (n.,%)	3 (25%)
Child Pugh stage (n, %) (among clinical cirrhosis)	
A	3 (100%)
MELD (among clinical cirrhosis) (median, IQR)	8 (6-12)
Antiviral therapy	
Paritaprevir-Ombitasvir-Ritonavir-Dasabuvir	5 (42%)
Sofosbuvir-Ledipasvir	3 (25%)
Sofosbuvir-Ribavirin	1 (8,3%)
Sofosbuvir-Daclatasvir	1 (8,3%)
Sofosbuvir-Velpatasvir	2 (16,7%)

to the lowest number of sequences per sample. The α-diversity indices were calculated by QIIME.

The taxonomy tables were imported into the R software (http: // www.r-project.org/) for statistical analyses and visualization. Non-parametric tests (Wilcoxon-Mann-Whitney test, Kruskal-Wallis nonparametric ANOVA) were used to evaluate differences in diversity indices or in specific taxa abundance in subjects grouped according to available categorical variables. All significance values were adjusted using the Benjamini-Hochberg correction to take into account the effect of multiple comparisons; a False Discovery Rate value <0.05 was considered statistically significant.

The study was conducted in compliance with the Declaration of Helsinki and the principles of good clinical practice. For this study informed consent was obtained from all subjects and/or their legal guardian(s). The study was approved by the "Federico II" Ethics Committee.

RESULTS

We enrolled 12 patients. The main demographic and clinical characteristics of the patients enrolled are shown in *Table 1*.

All patients enrolled (100%) achieved SVR12.

Microbial diversity tended to increase after treatment with DAAs (Direct Antiviral Agents) (*Figure 1*). Considering the microbiota composition, we found an increase in the abundance of Firmicutes and a decrease of Bacteroidetes after the treatment compared to baseline (*Figure 2*). Interestingly, potentially pathogenic microorganisms such as Enterobacteriaceae, Enterococcus, and Staphylococcus decreased upon treatment. (*Figure 2*) In detail, some taxa showed a

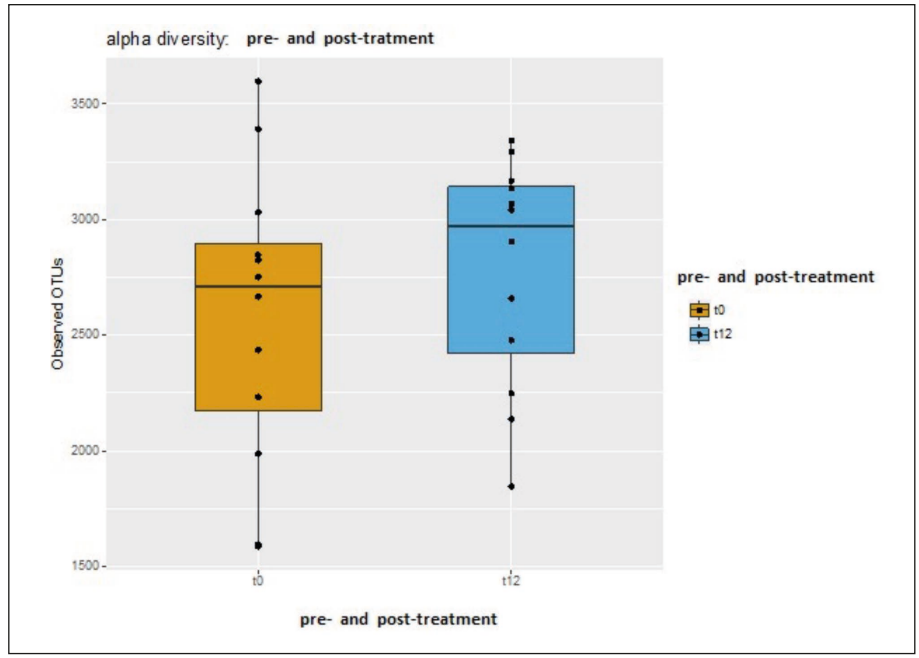

Figure 1 - *Number of observed OTUs in patients before (t0) and after 12 weeks of treatment (t12).*

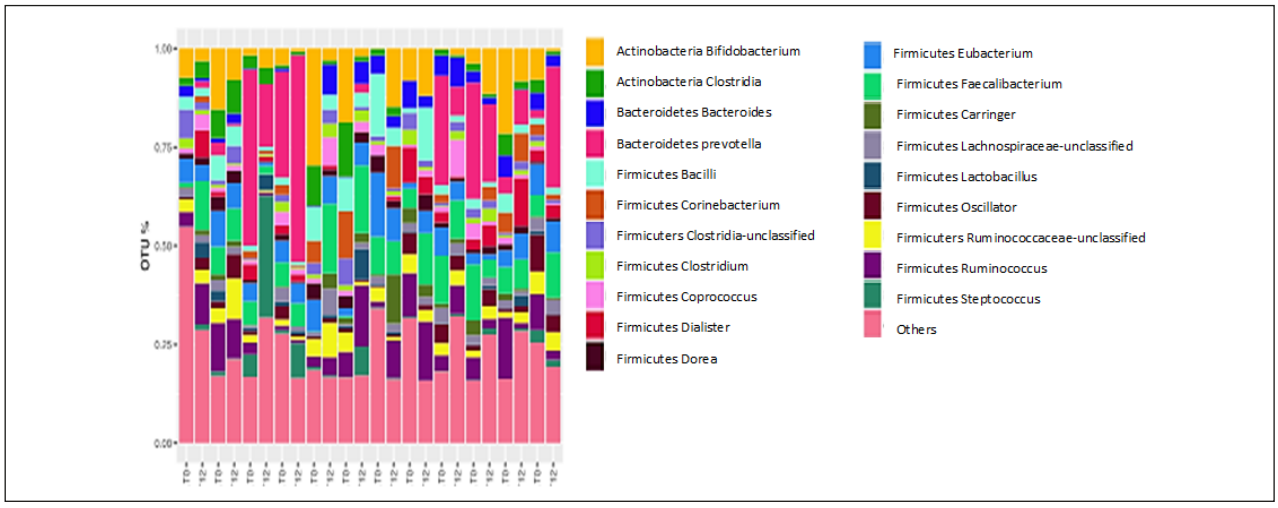

Figure 2 - *Bar-chart showing taxonomica composition of the microbiota at genus level of the 12 patients analyzed, pre- and post-treatment. T0: baseline - pre-therapy; T12: 12 weeks post-therapy.*

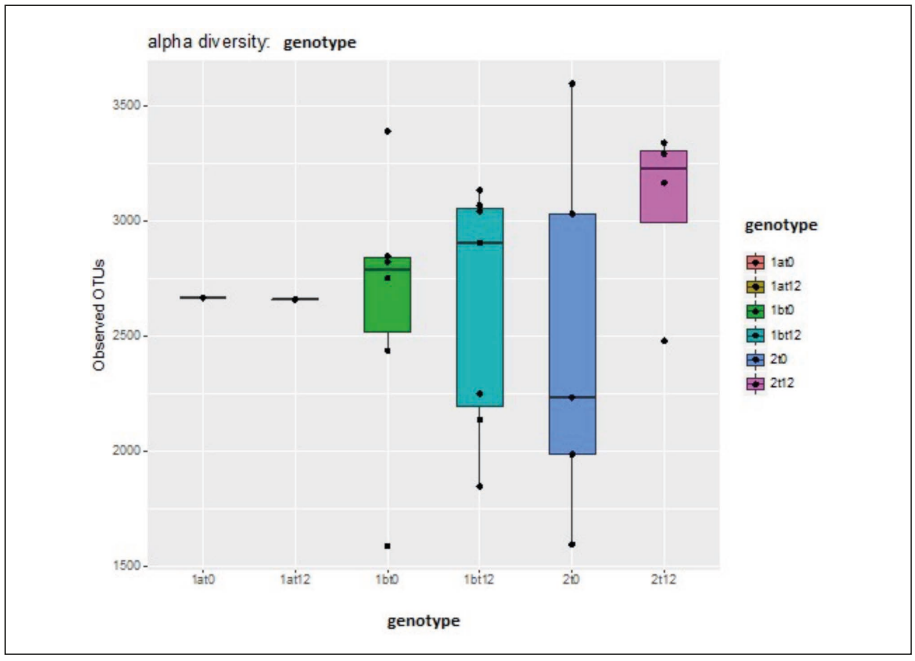

Figure 3 - *Variation of α-diversity in subjects grouped on the basis of the HCV genotype (1a, 1b and 2) and the 351 time of treatment (baseline, t0; 12-weeks, t12).*

change at the edge of statistical significance, namely, Bacillus (p: 0.08), Staphylococcus (p: 0.07), Aerococcus (p: 0.08), and Faecalibacterium (p:0.07).

The variation in microbial diversity was also evaluated in relation to viral genotype, presence/absence of cirrhosis, and type of treatment. The patients enrolled in the study were affected by genotype 1a, 1b, and 2. A trend towards an increase in microbial diversity was observed in patients with genotype 1b and 2 after therapy (*Figure 3*). Again, no statistically significant differences were observed. Moreover, we categorized the patients considering fibrosis stage and observed that in patients with fibrosis the eradication of the virus was associated with a trend toward an increase in microbial diversity, while this increase was less evident in subjects with cirrhosis (*Figure 4*). No statistically significant differences were observed by gender.

DISCUSSION

The new direct acting antivirals have constituted a real revolution in the treatment of HCV infection. This therapy has enabled the achievement of exceptional cure rates. Our study aimed to evaluate the change in the gut microbiota after viral eradication with DAAs. The results show a change in the gut microbiome upon achievement of SVR. We observed both a trend toward higher α-diversity and reduction

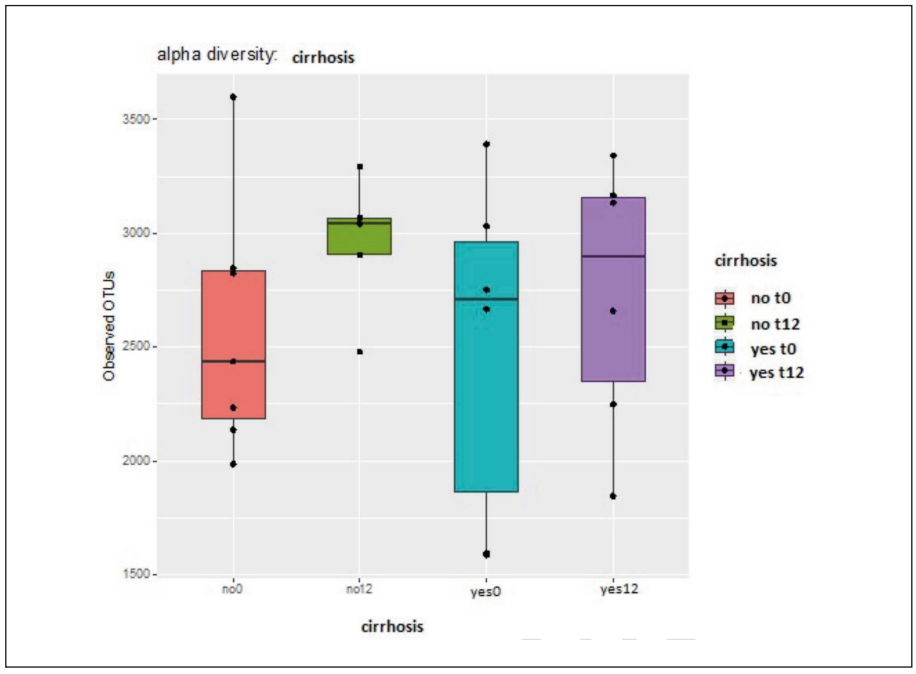

Figure 4 - *Variation of α-diversity in subjects grouped on the basis of the presence of cirrhosis (yes, with cirrhosis; no, without cirrhosis) and the time of treatment (baseline, t0; 12-weeks, t12).*

in the percentage of potentially pathogenic species. These results are in agreement with the study conducted by Chuaypen *et al.* (Wellhöner *et al*., 2021).
In particular, in patients with a low degree of fibrosis, the eradication of the virus corresponds to a marked trend of increase in gut microbial diversity, while in patients with cirrhosis, which can cause an imbalance in the microbiota by itself, the improvement after viral eradication was less evident. We underline that our results are similar to those obtained by Wellhoner *et al*., who found no changes in the gut microbiota of patients with liver cirrhosis after HCV eradication (Canani *et al*., 2017).
We acknowledge that our study has several limitations, i.e., the small sample size, the high proportions of patients with advanced fibrosis and cirrhosis over those with mild fibrosis. However, we underline that the changes in the gut microbiome observed in our study are biologically plausible, as they are more pronounced in patients without cirrhosis compared to those with cirrhosis. In the latter patients, in fact, the role of HCV infection should be considered one of the potential factors that may impair microbiome balance, together with the well-known systemic imbalance observed in patients with liver cirrhosis. The results of the present study may prompt other studies on this intriguing topic. In detail, aspects deserving further analysis include correlation of gut microbiome data with an evaluation of pre- and post-treatment inflammatory profile, biomarkers of liver fibrosis and gut-liver axis impairment. To our best knowledge, to date only 4 studies have evaluated changes in the gut microbiota during HCV infection. Bajaj *et al*. assessed the presence of an effective dysbiosis among patients with cirrhosis compared to healthy subjects. In addition, they evaluated the impact of viral eradication achieved with the old interferon-ribavirin combination regimen on microbiota without performing a longitudinal analysis of the pre- and post-treatment microbiota, comparing different subjects with or without virological response (Bajaj *et al*., 2016). The study by Aly *et al.* examined the microbiota of 6 Egyptian patients with HCV-related liver cirrhosis which presented less diversity of microbial species, and a relative abundance of Prevotella and Faecalibacterium compared with a group of healthy controls (Aly *et al*., 2017). Both studies conducted by Chuaypen N. et Wellhöner F. highlighted the positive impact of HCV eradication with DAAs on the gut microbiota. However, the study conducted by Wellhöner F. showed that such changes were evident only in patients without liver cirrhosis (Wellhöner *et al*., 2021; Dietrich *et al*., 2017).
In conclusion, our study shows that in the setting of HCV infection, viral eradication achieved via DAAs is associated with a trend of α -diversity increase and reduction in the percentage of potentially pathogenic species. Further studies with larger sample size are needed to confirm these data and to fully elucidate the potential effects of such gut microbiome changes.

Ethical approval and consent to participate
The study was conducted in compliance with the Declaration of Helsinki and the principles of good clinical practice. For this study, informed consent was obtained from all subjects and/or their legal guardian(s). The study was approved by the "Federico II" Ethics Committee with number 246/19.

Availability of data and materials

The data and materials are available upon request to the corresponding author.

Authors' contributions

P.B. participated in substantial contributions to the conception, design of the work, acquisition, analysis and interpretation of data for the work. S.R. conceived the idea with analysis and participated in interpretation of the literature, drafting the article, approving the final version to be published and is accountable for the accuracy/integrity of the content. Z.E. participated in revising the initial draft of the article and approving the final version to be. B.A.R. participated in drafting the article, and approving the final version to be published. M.A.E. participated in analysis and interpretation of data for the work. S.M.N. participated in the acquisition and analysis of data for the work. V.G. participated in design of the work and interpretation of data for the work. C.L. participated in approving the final version to be published and is accountable for the accuracy/integrity of the content. V.R. participated in analysis and interpretation of the literature, drafting the article, and approving the final version to be published. F.G. participated in revising the initial draft of the article and approving the final version to be published. D.F.F. participated in the acquisition and analysis of data for the work. E.D. participated in drafting the article, approving the final version to be published and is accountable for the accuracy/integrity of the content. G.I. participated in substantial contributions to the conception, design of the work, the acquisition, analysis and interpretation of data for the work, approving the final version to be published.

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