Characterization of the Intestinal Microbiome in Healthy Adults over Sars-Cov-2 Vaccination

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Abstract

Background: In response to the outbreak of coronavirus disease 2019 (COVID-19) worldwide, inactivated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines are implemented. Dysbiosis gut microbiota is implicated in the COVID-19 patients. Whereas, how intestinal microbiota are affected by vaccination remains elusive, and it is important to investigate the microbial shifts during vaccines treatment. Methods: In the present study, we assessed the gut microbial composition in healthy adults, and performed comparison before and post an inactivated SARS-CoV-2 vaccine candidate, BBIBP-CorV vaccination. Results: Microbial diversity in shannon, pielou evenness, simpson and invsimpson index was remarkably suppressed by vaccination. Potential functional profiles of gut microbiome in amino acid metabolism, lipid biosynthesis proteins and steroid biosynthesis were remarkably increased, while the capacity in renin-angiotensin system was remarkably decreased following vaccines.

Conclusions: Our study suggests that inactivated BBIBP-CorV against SARS-CoV-2 could elicit modulations on gut microbial composition and functions, which might favor host immune response and protect from COVID-19.

Keywords: gut microbiota; COVID-19; SARS-CoV-2; vaccine; BBIBP-CorV

1. Introduction

Since the rapid spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection across the globe, the persistent prevalence of coronavirus disease 2019 (COVID-19) has continuously brought on a worldwide public health crisis, and devastating global toll [1,2]. There have been 593,269,262 cases of COVID-19 confirmed globally, with 6,446,547 deaths recorded as of August 22, 2022 [3]. The symptoms of COVID-19 are demonstrated to be primarily in the respiratory system, as well as extra-pulmonary manifestations such as gastrointestinal symptoms, thrombotic complications, and acute kidney injuries etc. [4]. Researchers from multiple regions of the world have indicated that 15%–69% of patients with COVID-19 showed abnormal gastrointestinal symptoms [5–8]. To control the COVID-19 pandemic, lower the infection rates and minimize serious cases, highly efficacious vaccines targeting SARS-CoV-2 were urgently developed [9]. The inactivated vaccine BBIBP-CorV (Sinopharm) with potent protection against SARS-CoV-2, have been validated to be safe and well tolerated in randomized, double-blind, placebo-controlled, phase 1/2 trials [10–12]. And at present, BBIBP-CorV has become one of the most predominant vaccine injections in China.

Mounting evidence suggested that the intestinal microbiome played a pivotal in physiological and pathological status of the host, including COVID-19. Hallmark features of the gut microbial ecology have been demonstrated to be distorted in SARS-CoV-2 infected patients, even in asymptomatic very young children [13,14]. The loss of beneficial bacteria and bloom of opportunistic fungi investigators observed post COVID-19, are speculated to be largely a consequence of SARS-CoV-2 infection [13]. More than that, alterations of the gut microbiota with impaired functional profiles were correlated with COVID-19 disease severity in both human and experimental animals [15,16]. The onset of complications and mortality due to COVID-19 were also demonstrated to be implicated with gut bacterial dysbiosis and instability [17].

Considering these findings, a growing number of reports are indicating that microbiota modulation might facilitate improvement and recovery from COVID-19 syndrome. A randomized, quadruple-blinded, placebo-controlled trial showed that probiotic formula consisted with strains Lactiplantibacillus plantarum plus Pediococcus acidilactici reduced nasopharyngeal viral load and lung infiltrates in COVID-19 patients [18]. Another symbiotic formula of Bifidobacteria strains etc. derived from gut microbiota, was confirmed to restore microbial dysbiosis, hasten anti-
body formation against SARS-CoV-2, reduce nasopharyngeal viral load and pro-inflammatory immune markers in COVID-19 patients [19]. In addition, metabolites identified from human microbiome commensals were proved to be with SARS-CoV-2 inhibitory activity [20]. As intestinal microbiota plays a critical role in host immune response and COVID-19, it was suggested most recently that, specific gut microbiota markers were in association with improved immune response and reduced adverse events following SARS-CoV-2 vaccines, such as inactivated vaccine (CoronaVac; Sinovac) or the mRNA vaccine (BNT162b2; BioNTech; Comirnaty) [21]. Nevertheless, direct evidence for the probable correlation of an inactivated SARS-CoV-2 vaccine candidate, BBIBP-CorV (Sinopharm) and gut microbiome remains scarce.

Hence, to elucidate whether intestinal flora is involved in BBIBP-CorV-induced protective response, we investigated in the current study that, the shifts of the gut microbiome in human adults before and post BBIBP-CorV treatment.

2. Materials and Methods

2.1 Study Participants

We collected fecal samples from fourteen healthy adults, who were traced since January to May 2021 at Beijing Chaoyang Hospital. Each volunteer was examined at the baseline before subjecting to injection of BBIBP-CorV vaccines (Before group, n = 14), and after they finish the completely two doses intramuscular injection of vaccines (After group, n = 14). The samples were harvested twice from each enrolled participant at two weeks prior to injection and two weeks after receiving the vaccines, when the vaccines was considered to be effectively activate the host immune system. Individuals with or with a history of cancer, heart failure, renal failure, stroke, peripheral artery disease, and chronic inflammation disorders were excluded. Moreover, pregnant or breast-feeding female subjects were excluded. Other exclusion criteria were subjects with previous SARS-CoV-2 exposure or infection, and those who received statin, aspirin, insulin, metformin, antibiotics, or probiotics etc. within the last two months. In present study, a self-controlled protocol was designed to eliminate some confounding factors including Body Mass Index (BMI), smoking status, alcohol consumption, dietary habits, and physical exercises etc. And food habits and lifestyle behaviors did not change significantly during the short survey periods. This study was approved by the Medical Ethics Committee from Beijing Chaoyang Hospital and carried out in accordance with the Helsinki declaration. Written informed consent was obtained from all the participants.

2.2 Fecal DNA Extraction and 16S rRNA Sequencing

The middle section of fresh fecal samples was collected from participants, and immediately stored in a freezer at –80 °C within half hour. Fecal microbial DNA was extracted with a TIANamp Stool DNA Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer’s instructions. Isolated DNA samples were quantified using a NanoDrop® ND-2000c UV-vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and DNA integrity was estimated with agarose gel electrophoresis. Fecal microbial DNA samples were diluted to 1 ng/µL and stored at –20 °C for further examination. The V3–V4 region of bacterial 16S rRNA gene was amplified through Polymerase chain reaction (PCR) with universal primer set 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’). Following library preparation and generation, then we assessed the library quality on the Qubit® 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system. The libraries were subsequently sequenced on IlluminaHiSeq instrument 2500 and 250 bp paired-end reads were obtained.

2.3 Operational Taxonomic Units (OTUs) Clustering and Taxon Annotation

According to the information of primers and unique barcode regions, sequencing data for each sample was assigned. By removing the barcode and primer, all pair-end sequences were merged with FLASH (Version 1.2.17, Baltimore, MD, USA) to obtain the raw tags. Strict quality-controlled processes including truncating tags with continuous low-quality bases and filtering out tags with <75% high quality bases were carried out with QIIME (Version 1.9.1, Denver, CO, USA). Effective clean tags were acquired when chimeras were further removed using UCHIME algorithm (Version 7.0.1001, Tibruron, CA, USA). Utilizing UPARSE (Version 7.0.1001, Tibruron, CA, USA), effective tags from all samples were constructed into OTUs, with a cutoff for aligning identity at 0.97. The phylogenetic affiliations of clustered OTU sequences were annotated through RDP classifier (Version 2.6, East Lansing, MI, USA) with a threshold at 0.8. Sequences failed to be assigned into specific taxonomy were considered as unclassified.

2.4 Alpha Diversity of Gut Microbiome

Rarefaction analysis and OTUs accumulation curves by randomly including certain number of individuals and counting the observed OTUs were applied to evaluate the rationality of sample size. And rarefaction curves by gradually increasing the sequencing depth were conducted to examine the rationality of sequencing quantity. Bacterial alpha-diversity based on OTU profiles was presented by the chaot richness, Shannon diversity, pielou evenness, ACE index, simpson index and invsimpson (inverse simpson) index using the R software (Version 3.3.3, Auckland, New Zealand) program package “vegan”. Significant differences of alpha-diversity indices in each individual before and after vaccination were tested by Wilcoxon matched-pairs signed rank test.
2.5 Beta Diversity Analysis of Gut Microbiota

Subsequently, the relative abundance of OTUs and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KOs) profiles was applied to generate the bray-curtis distance through QIIME (Version 1.9.1, Denver, CO, USA) and assess the microbial beta-diversity. Non-metric dimensional scaling (NMDS), principal-component analysis (PCA) and principal coordinate analysis (PCoA) were conducted with the vegan package, ade4 package and WGCNA package in the R software (Version 3.3.3, Auckland, New Zealand), respectively. Significant differences between groups were tested by Analysis of similarities (Anosim).

2.6 Functional Annotation

According to the KEGG database, the potential functional capabilities of gut microbiota were predicted and identified by phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) Version 1.0.0 (Halifax, NS, Canada). KEGG ontology and pathway profiles were generated based on the OTU tree and the gene information in Greengene database. Predicted functional genes were categorized into KO, and assign into KEGG pathway.

2.7 Statistical Analysis

Analysis for the demographic and clinical data was conducted with the SPSS 20.0 statistical package (IBM, IL, USA), using two-tailed student’s t-test or chi-square test, as appropriate. Significant differences of alpha-diversity indices were tested by Wilcoxon matched-pairs signed rank test, and of beta-diversity were determined by Anosim. The Differential abundance of taxa and KOs for paired samples (two samples before and after treatment from the same patients) was determined by the Wilcoxon matched-pairs signed rank test. \( p < 0.05 \) was set as statistically significant.

3. Results

3.1 Clinical Characteristics of the Recruited Subjects

The characteristics of 14 healthy adults included in this study were summarized in Supplementary Table 1. The mean age of the study population is 38.4 years, and 71.4% were men. Meanwhile, the mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the participants are 120.7 and 73.9 mmHg, respectively.

3.2 Gut Microbial Diversity Varied Following Supplementation with BBIBP-CorV Vaccines

Twenty-eight fecal samples from all the recruited subjects were sequenced on an IlluminaHiSeq sequencer. A total of 1,970,533 qualified tags were obtained from 2,139,298 raw reads, and for each sample, 65,364 effective tags were acquired. A total of 85.41% of all qualified tags were clustered into qualified OTUs. OTUs those detected in less than 2 samples were discarded for their low frequencies in samples. Ultimately, qualified operational taxon-
Fig. 2. Characterization of the phylogenetic profiles in the gut microbiome of recipients before and after vaccination. (A) Average composition of the bacterial community at the phylum level was compared before and after vaccines treatment. (B) Relative abundance of the top 10 bacterial phyla in the fecal samples from each participant who underwent vaccination. (C) Average abundance of the top 10 genera was compared before and after receiving vaccines. (D) Relative composition for the top 10 bacterial genus in the fecal samples of each participant before and after vaccination.

omy units (OTUs) were clustered for downstream analysis. The details for sequencing characteristics are shown in Supplementary Table 2 of the supporting information.

Rarefaction analysis was carried out and OTUs accumulation box plots were performed to describe increased diversity and richness following expanding the sample size. The rate at which novel OTUs emerge by continuous sampling was estimated based on the box plots in Supplementary Fig. 1A. It was observed to be tending flat, but not sharply increased, indicating that the OTUs would not significantly augment with larger sample size. On the other hand, by randomly extracting a certain amount of sequencing data from the samples, the number of OTUs they represent was depicted with rarefaction curves (Supplementary Fig. 1B). It reflected that the sequencing depth approached stable, and elevated sequencing data amount will generate only a few new OTUs.

The richness of observed OTUs was comparable in participants before and after vaccines treatment (Fig. 1A). In addition, parameters related to alpha-diversity including chao1 richness, shannon diversity, pielou evenness, ACE index, simpson index and invsimpson index were accessed (Fig. 1B–G). We found that both chao1 and ACE were similar between the two groups prior to and post receiving vaccines. Interestingly, the results showed that intestinal microbial alpha diversity in shannon diversity, pielou evenness, simpson index as well as invsimpson index was remarkably reduced in recipients after vaccination than before vaccination, indicating that bacterial homeostasis was affected by vaccines. The beta diversity results based on NMDS, PCA and PCoA plots showed that there was no significant distinction in fecal microbial community distri-
bution between groups (Supplementary Fig. 2). Consequently, it was suggested that BBIBP-CorV vaccines impacted gut microbiome by eliciting depressed microbial alpha-diversity.

### 3.3 Fecal Microbiota Taxonomic Composition in Human Volunteers is Affected by Vaccination

Having established that microbial alpha-diversity differed significantly by vaccines taxonomically, we next examined phylogenetic profiles in the gut microbiome and investigated the taxonomic composition alterations associated with vaccines treatment. Relative abundances of bacterial phylum and primary genus in the fecal samples from subjects who underwent vaccination were revealed. Firmicutes, Bacteroidetes, Verrucomicrobia and Proteobacteria were the most dominant phylum identified. Before vaccination, the proportions of Bacteroidetes and Proteobacteria were higher in their relative bacterial abundances. By contrast, the proportions of Firmicutes were more enriched after vaccination (Fig. 2A,B). In addition, at the genus level, Faecalibacterium, Bacteroides, Blautia, Prevotella, Bifidobacterium, Lachnospira and Roseburia were the most abundant entities in the gut microbiota. After vaccination, the subjects exhibited enriched Faecalibacterium, Bifidobacterium, Lachnospira and Roseburia in terms of relative bacterial abundance, but less abundance of Bacteroides and Blautia (Fig. 2C,D).

Further, we performed differential expression analysis of bacteria abundance between Before and After vaccines inoculation groups through Wilcoxon matched-pairs signed rank test. At the phylum level, Verrucomicrobia was observed to be decreased with marginal significant after vaccination (Fig. 3A). We found that the classes of Coriobacteria, Verrucomicrobiae and order of Coriobacteriales, Verrucomicrobiales were reduced marginal significantly, whereas Betaproteobacteria class and Burkholderiales order elevated after receiving the vaccines (Fig. 3B,C). In addition, decreased abundance of the family Verrucomicrobiaceae, Coriobacteriaceae, Peptostreptococcaceae, Mogibacteriaceae, but enhanced Alcaligenaceae was detected following vaccines treatment (Fig. 3D). The Actinomycetales order and two families including Oxalobacteraceae and Actinomyctecaceae were prominently decreased after vaccination (Fig. 3C,D). Among them, 12 taxa were depleted and 3 were enhanced by vaccines (Fig. 3E). At the genus level, Collinsella and Akkermansia genera were reduced, while Roseburia and Sutterella genera were overgrowth after vaccines treatment (marginal significant) (Fig. 4A). It was intriguingly that Ruminococcus and Actinomyces were strikingly deficient after vaccination, with fold change at 0.52 and 0.31, respectively (Fig. 4A,B). And Faecalibacterium was dramatically augmented with 1.58-fold changes in recipients of vaccines (Fig. 4A,B).

### 3.4 Function Changes Associated with BBIBP-CorV Vaccines Treatment

To predict the microbial community function profiles, all the observed OTUs were aligned into the PICRUSt built-in reference database. Therefore, a total of 5661 KOs were parsed and mapped into 276 KEGG pathways. Functionally, we again failed to identify obviously distinct clusters between individuals before and after vaccination based on NMDS, PCA and PCoA plots of Bray-Curtis pairwise dissimilarities (Supplementary Fig. 3). Nevertheless, the top 30 most significantly affected KOs and a total of 17 prominently altered KEGG pathways by vaccination were presented in the heat map with abundance (Fig. 5A,B). There were 6 KOs suppressed and 24 KOs activated by vaccines, including branched-chain amino acid transport system and glutamate synthase (NADPH) small chain (Fig. 5A). Among the 17 KEGG pathways identified to be with significant differences between Before and After group, 10 functions, such as amino acid metabolism, lipid biosynthesis proteins and Steroid biosynthesis, were remarkably increased, while 7 functions, such as reninangiotensin system and biotin metabolism, were remarkably decreased following vaccines treatment.

### 4. Discussion

The microbial communities that reside in the human gastrointestinal tract are important for developing and maintaining the host immune homeostasis. Accumulating evidence has demonstrated that the gut microbiome was perturbed, and microbial composition was significantly varied among COVID-19 patients, characterized by augmented opportunistic pathogenic species. Furthermore, the disease severity-related microbial features of COVID-19 were identified to be related to host immune response [22,23]. Consequently, microbiota-derived symbiotic formula was applied to COVID-19 patients, and it was observed to boost immunity against COVID-19 by hastening antibody formation, reducing nasopharyngeal vital load and pro-inflammatory response markers [19].

Vaccination as an essential strategy in the fight against COVID-19 was further focused. Both the oral and intestinal microbiome have been suggested to be altered after receiving SARS-CoV-2 vaccines [21,24]. For individuals subjected to different types of vaccines, the alterations of their intestinal microbiome were not quite similar. Ng SC et al. [21] have performed shotgun metagenomic sequencing on stool samples of 37 individuals receiving inactivated vaccine (CoronaVac) and 101 inoculated with mRNA vaccine (BNT162b2). It was observed that Bacteroidesascaccae was persistently higher in CoronaVac group, while subjects in BNT162b2 group showed increased B. caccae and Alistipesshahii. On the other hand, declined Adlercreutziaaequivalenti and A saccharobactercelatus etc. were detected under both CoronaVac and BNT162b2 treatment [21]. In addition, the proportion of the oral bacteria genus
Fig. 3. Comparison of the relative abundance in fecal taxa between samples obtained from recipients before and after vaccination. (A) Gut phylum Verrucomicrobia displayed marginal statistical difference after vaccination. (B) Classes with marginal statistical difference following vaccination. (C,D) Orders and families shifted in gut microbiome after receiving vaccines treatment. The horizontal lines within the box plots were medians; the upper and lower ranges of the box represent 75% and 25% quartiles, and dot represents each sample. \( p \) values were obtained from Wilcoxon matched-pairs signed rank test. (E) The enrichment and depletion of gut taxa, and fold changes for their alterations following vaccination were illustrated in heat map.
Fig. 4. Comparison for the abundance of genera in fecal taxa between samples obtained from recipients before and after vaccination. (A) Intestinal bacteria at genus level were identified to be altered following vaccines treatment. The horizontal lines within the box plots were medians; the upper and lower ranges of the box represent 75% and 25% quartiles, and dot represents each sample. $p$ values were obtained from Wilcoxon matched-pairs signed rank test. (B) The enrichment and depletion of gut genera, and corresponding fold changes after vaccination.

*Bacteroides* was significantly decreased post SARS-CoV-2 mRNA vaccination. Nevertheless, the probable relationship between BBIBP-CorV inactivated vaccine and gut microbiome remains unknown. And in our study, the alterations of intestinal microbiome following BBIBP-CorV inactivated vaccine were monitored.

Gut microbial diversity has been known to reflect the number and variety of intestinal bacteria, which were frequently associated related with disease states [25]. Previous studies suggested that, when compared with healthy populations, significantly reduced bacterial diversity was observed among COVID-19 patients [26]. Interestingly, our findings in the present work showed that, the alpha diversity indices of gut microbiome, such as shannon diversity and pielou evenness, were remarkably decreased after subjected to BBIBP-CorV vaccination. It was thus indicated that the gut microbial homeostasis was possibly affected by vaccines, which is consistent with previous findings observed by Ng SC et al. [21], and supported the connections among vaccines, SARS-CoV-2, and gut microbiota. Furthermore, phylogenetic profiles of the gut microbiome were investigated to evaluate the alterations in taxonomic composition caused by vaccines treatment. In a holistic perspective, the proportion of several groups of short-chain fatty acid-producing bacteria [27], for example the genus *Roseburia* and *Faecalibacterium*, were increased after BBIBP-CorV vaccination. At the genus level, both *Ruminococcus* and *Actinomyces* were reduced, whereas *Faecalibac-
Fig. 5. Functional capability of gut microbiome varied dramatically in individuals after receiving vaccines. (A) Heat map showing the top 30 most significantly fluctuated KEGG orthologs (KOs) by vaccination, with 6 KOs reduced and 24 KOs enhanced after vaccination. (B) There were seventeen KEGG pathways at level 3 identified to be prominently affected by BBIBP-CorV vaccines. The abundance profiles are transformed into Z scores by subtracting the average and dividing the standard deviation of all the samples. Z score is negative in blue when the row abundance is lower than the mean, and positive in orange when the row abundance is higher than the mean.

*Actinomyces* were overgrowth in individuals post BBIBP-CorV vaccine treatment. It was intriguingly that, the gut bacteria *Actinomyces* has been documented to be significantly increased, while genera *Faecalibacterium* to be dramatically depressed in COVID-19 patients [26]. In our present study, the significant decrease in genera derived from Firmicutes and Actinobacteria could be explained by altered physiological functions and intensive inflammation during vaccination [28]. We hence speculated that, the BBIBP-CorV vaccines might alter the proportion and abundance of specific intestinal microbiota to prevent or resist the possible adverse effects of SARS-CoV-2 in advance.

Along with the shifts in microbial compositions, gut bacterial functions in KOs and KEGG pathways were detected to be varied after vaccination. The alterations of the top 30 most significantly affected KOs, and 17 KEGG pathways were identified to be induced upon BBIBP-CorV vaccination. Previous study showed that two repurposed drugs that imatinib and methazolamide, could act as angiotensin converting enzyme 2 (ACE2) enzymatic activators to potentially improve glucose and lipid metabolisms after SARS-CoV-2 infection [29]. Interestingly, in the present study, several KOs and KEGG pathways associated with lipid metabolism, such as lipid biosynthesis proteins and steroid biosynthesis, and glucose metabolism, for instance glutamate synthase (NADPH) small chain, were significantly enriched post vaccination, which might be characteristics in the vaccinators.
To our knowledge, the present study was the first effort to clarify how inactivated SARS-CoV-2 vaccine BBIBP-CorV affects the composition of gut microbiome, and our findings might provide essential information for the variations of intestinal environment upon the immune system’s response to inactivated SARS-CoV-2. Furthermore, this work enabled us to exploit novel management strategies targeting intestinal health to combat COVID-19. However, there are also several limitations. Firstly, the sample size is relatively small. Here, rarefaction curves were performed to evaluate the sufficiency of sample size in each group. We found that the curves tend to be flat, which means that there would be only a handful of new species characteristics yielded when including more samples. And further investigations with larger sample size are still necessary to deeper validate and confirm our findings in the present study. Moreover, samples from a single time point post vaccination were collected and examined, and the possibility of dynamic changes of gut microbiome cannot be ruled out. Further investigations with fecal samples collected at continuous time points after vaccination could provide potential mechanisms for more reliable and detailed gut microbial response to COVID-19 vaccines.

5. Conclusions
In the current study, we demonstrated that inactivated BBIBP-CorV against SARS-CoV-2 modulated intestinal bacterial diversity and gut microbial composition, including dramatically reduced the microbial diversity, increased the abundance of Faecalibacterium, along with depressed level of Ruminococcus and Actinomyces in the same individuals post BBIBP-CorV treatment. Also, the findings regarding microbial functional profiles indicated that amino acid metabolism, lipid biosynthesis proteins and steroid biosynthesis were increased, while the capacity in renin-angiotensin system was remarkably decreased after vaccination. Taken together, these results revealed the shifts in gut microbial composition and functions within recipients of inactivated BBIBP-CorV vaccines, which might modulate the host immune responses and protect against COVID-19.

Data Availability

Data sets used and analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions
JJ, YS, YD and JL conceived the study, directed the project, designed the experiments; JL, YD, JJ and YS interpreted the results and wrote the manuscript; PW, KZ, XY, MC recruited and collected the clinical details from the subjects; JL, YD, JJ and YS analyzed the data. JL and YD revised the manuscript.

Ethics Approval and Consent to Participate
This study was approved by the Medical Ethics Committee from Beijing Chaoyang Hospital and carried out in accordance with the Helsinki declaration. Written informed consent was obtained from all the participants.

Acknowledgment
Not applicable.

Funding
This research received no external funding.

Conflict of Interest
The authors declare no conflict of interest.

Supplementary Material
Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2710280.

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