Translational Medicine Communications

PROTOCOL

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The gut microbiota patterns of patients with COVID-19: protocol for a case-control study

Seyed Aria Nejadghaderi[1,2,3], Ehsan Nazemalhosseini-Mojarad[4], Asra Fazlollahi[5,6], Ali Pirsadeghi[7,8], Ainaz Khalili[9], Rasoul Ebrahimi[3] and Nima Rezaei[10,11,12*]

Abstract

Background SARS-CoV-2 caused an outbreak in late December 2019. It has been suggested that gut microbiota dysbiosis influences the severity, mortality, and quality of life of patients with COVID-19. So, identifying the gut microbiota pattern could be helpful to determine the prognosis of the disease, and maybe determine some potential treatment approaches. Our aim will be to compare gut microbiota patterns between patients with severe or non-severe COVID-19, and healthy controls.

Methods We will include 183 samples: 122 samples from COVID-19 patients, including 61 severe patients and 61 non-severe patients, and 61 samples from healthy controls. Total bacterial DNA will be extracted from samples and 16 S rRNA gene will be amplified through two polymerase chain reaction (PCR) stages. Fecal samples will be analyzed using a targeted metabolomics technique. The differences in each RNA or DNA expression between patients with severe COVID-19, patients with non-severe COVID-19, and controls will be compared. Also, we will assess the relationships between each DNA or RNA and the risk of COVID-19 severity, sort of clinical manifestations, and comorbidities. Concurrent medication data will be collected and patients will also be grouped based on their drug history.

Results We hypothesize that the gut microbiota composition will be affected by the COVID-19 severity and there might be differences in terms of sex and age.

Conclusions The results of our study could be the backbone for further trials which might lead to the development of prognostic factors and treatment options. Further studies can also consider the limitations of the study like potential confounders and selection and recall biases.

Keywords Gut microbiota, Dysbiosis, COVID-19, SARS-CoV-2

*Correspondence:
Nima Rezaei
rezaei_nima@tums.ac.ir; rezaei_nima@yahoo.com

Full list of author information is available at the end of the article

Introduction

In late December 2019, a new species of coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan City, Hubei Province, China [1]. It rapidly spread to almost all countries and territories worldwide and the severe cases increased. As a result, the World Health Organization declared the coronavirus disease 2019 (COVID-19) as a pandemic on March 11, 2020 [2]. Since then, the social and public health burden of the disease has been inferred to be tremendous [3]. As of July 2023, more than 770 million confirmed cases and 6.9 million deaths have been reported globally [4].

There are now two main groups of drugs used to treat SARS-CoV-2. Repurposed and experimental treatments that aim to get involved at different points in viral entrance and replication fall under the first group. These interventions include steps like suppressing viral RNA polymerase (e.g., ribavirin), blocking protease activity (e.g., lopinavir and ritonavir), and preventing the glycosylation of host cell receptors. The second group includes supplementary medicines, such as convalescent plasma therapy and monoclonal antibodies that target interleukin-6 (IL-6) [5]. Not only do the above mentioned medicines have a variety of gastrointestinal (GI), hematologic, cardiovascular, and neurologic adverse events, but also they have some contraindications like pregnancy and hemoglobinopathies [6]. So, it is necessary to try to find safer and more efficient treatments.

The human gut microbiome mostly includes *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria, and Verrucomicrobia* phyla, of which the first two ones have the highest frequency [7, 8]. Moreover, evidence showed that sex and age can affect the gut microbiota pattern of human populations, in which females, especially those above the age of 50 years have a significantly higher Firmicutes to Bacteroidetes ratio than males [9]. The changes in the sexual hormones between males and females in different age groups, for example, testosterone peak in puberty or postnatal period in males, can adjust the variations [10]. The gut microbiota imbalance can potentially contribute to the development of some pathological conditions, such as pancreatic diseases [11], irritable bowel syndrome [12], ulcerative colitis [13], Parkinson's disease, and amyotrophic lateral sclerosis [14]. Also, there is impressive evidence suggesting that the gut microbiota influences the occurrence of some respiratory diseases like asthma or chronic obstructive pulmonary disease [15]. Supporting this hypothesis, Hilty et al. showed that the asthmatic subjects were distinguished by excess of *Haemophilus spp.* and *Staphylococcus spp.*, while *Prevotella spp.* were more common in the control group [16].

There are some ongoing clinical trials that aim to evaluate the relationship between fecal microbiota composition and severity, mortality, and quality of life of patients with COVID-19 [17, 18]. A pilot study on 15 patients with COVID-19 revealed that antibiotic-naive COVID-19 patients had reduced numbers of some bacterial species, including *Fecalibacterium prausnitzii*, *Eubacterium rectale*, *Ruminococcus obeum*, and *Dorea formicigenerans*, in comparison with patients received empirical therapy [19]. Furthermore, *Firmicutes* phylum has the highest correlation with the severity of COVID-19, which seems to be a result of the effects of the bacteria of this phylum on alterations in angiotensin-converting-enzyme 2 expression [19].

Currently, different vaccines with suitable levels of immune responses have been developed for COVID-19 [20]. However, it is necessary to be administered two or three times and also repeated periodically [20]. Additionally, they have different adverse events like neurological complications, myocarditis, and Bell's palsy [20]. Although the development of vaccines led to the prevention and control of COVID-19, severe cases of COVID-19 still occur, especially in individuals with underlying diseases and immunosuppression. As a specific safe and effective treatment option for COVID-19 has not been developed yet, other new potential treatment options need to be evaluated. Also, the use of fecal microbiota transplantation for COVID-19 has been proposed as a potential treatment strategy [21]. Previous research has aimed to explore the association between gut microbiota and COVID-19, but some of these studies have reported limitations like the heterogeneity of patient clinical management [22], the limited number of cohorts [23], or being under medical treatment [24] that must be considered. This study aims to compare the gut microbiota patterns between severe COVID-19 patients, non-severe COVID-19 patients, and healthy controls. The results of the present study might help to consider the modulation of the gut microbiota as a potential treatment target for COVID-19.

Methods

Study design

This will be a single-center case-control pilot study on hospitalized COVID-19 patients and healthy controls. We will include 183 samples, using a convenience sampling method. Among them, 122 samples will be from COVID-19 patients (61 severe and 61 non-severe patients) and 61 samples will be from healthy controls. The data regarding lifestyle (smoking, stress), diet, and demographic factors, including age and sex, will be collected and participants will be matched based age and sex. Also, comorbidities, clinical manifestations of COVID-19, and stool microbiota levels will be collected

Table 1 A template table for presenting the main characteristics of included participants

Parameters	Patients		Controls
	Severe	Non-severe	
Sex			
Male			
Female			
Age (year)			
18–40			
41–60			
> 60			
Drug history			
Comorbidities			
Hypertension			
Diabetes mellitus			
Hyperlipidemia			
Coronary artery disease			
Dementia			
Respiratory diseases			
Malignancy			
Atrial fibrillation			
Heart failure			
Immunodeficiency states			
Co-existing infections			
Renal disorders			
Life style			
Smoking (pack/year)			
Stress			
Diet			
Diet quality			
Diet diversity			
Compliance with a Mediterranean diet			
Clinical manifestations			
Cough			
Dyspnea			
Fever			
Myalgia			
Headache			
Sore throat			
Diarrhea			
Nausea/vomiting			
Loss of smell or taste			
Abdominal pain			
Rhinorrhea			
Conjunctivitis			
Dermatologic findings			
Fecal microbiota composition			
Firmicutes			
Bacteroidetes			
Proteobacteria			
Actinobacteria			
Verrucomicrobia			
Others			

(Table 1). We will also collect the data regarding the drug history and concomitant medications of the patients and the grouping of patients will be based on the drugs they have used.

Participants

We will include participants aged between 18 and 70 years with real-time-polymerase chain reaction positive for SARS-CoV-2 and not currently residing in an institution, such as a prison, nursing home, or shelter. Our exclusion criteria will be the following: (1) disturbance of consciousness; (2) difficulty in swallowing and frequent vomiting; (3) requiring blood transfusion; (4) infection with fungus or other identified pathogens; (5) pregnancy or lactation; (6) refusal to sign informed consent form; (7) history of bariatric surgery, total colectomy with ileorectal anastomosis or proctocolectomy; (8) postoperative stoma, ostomy, or ileoanal pouch; (9) treatment with total parenteral nutrition; (10) regular use of probiotic or antibiotic within two weeks before entering the study; and (11) other conditions that the investigator considers ineligible for the study.

For the control group, the participants should have a negative real-time-polymerase chain reaction test for SARS-CoV-2 and will be recruited from the same center where cases have been selected. Also, those who have a regular use of probiotics or antibiotics within two weeks before entering the trial, those with medical history of GI diseases, surgery (e.g., bariatric surgery, total colectomy with ileorectal anastomosis or proctocolectomy), and fecal/gut microbiota transplantation, those refused to participate in the study, those with underlying diseases (e.g., human immunodeficiency virus infection and tuberculosis), and pregnant and lactating women will be excluded.

Outcomes

Our primary outcome will be to identify the gut microbiota patterns of patients with SARS-CoV-2 infection compared to healthy controls.

The secondary outcomes will be:

1. To evaluate the relationship between fecal microbiota composition and the severity of COVID-19;
2. To investigate the association between the types of clinical manifestations of COVID-19 and the fecal microbiota composition;
3. To determine the differences in fecal microbiota composition of COVID-19 patients by sex and age; and.
4. To analyze the effects of the numbers of gut microbiota organisms in the severity of COVID-19.

Controls and patients

Before starting any treatment, stool samples will be taken from 122 newly diagnosed COVID-19 cases, including 61 severe and 61 non-severe patients, and 61 healthy controls. Severe cases are defined as patients who have oxygen saturation below 94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen < 300 mmHg, a respiratory rate above 30 breaths/minute, or lung infiltrates > 50% [25]. Medical records or direct questions to participants will be used to gather clinical data. Each participant will provide a sample of 50 mg of feces, which will be put into a tube for processing of the sample and analysis. Each patient's 1 µL of serum will be examined utilizing proteomics technology. The serum will first be denatured for 30 min at 30 °C in 20 µL of buffer containing 8 M urea (Sigma, 444 #U1230) in 100 mM ammonium bicarbonate. After being alkylated with 40 mM iodoacetamide (IAA, Sigma, #SLCD4031) in complete darkness for 45 min, the lysates will be reduced with 10 mM tris (2-carboxyethyl) phosphine (TCEP, Sigma #T4708) at room temperature for 30 min. After being diluted with 70 µL of 100 mM ammonium bicarbonate to ensure that the urea concentration is less than 1.6 M, the solution will undergo two rounds of tryptic digestion at 32 °C for 4 h and 12 h, respectively, using 2.5 mL of trypsin (0.4 g/L) in each stage. The process will then stop by acidifying the solution with 1% trifluoroacetic acid (TFA; Thermo Fisher Scientific, #T/3258/PB05) to a pH of 2–3. C18 will be used to clean peptides (Thermo, #60209-001).

Microbiome analysis

DNA extraction

Following the manufacturer guidelines, total bacterial DNA will be extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany). The Qubit quantization system will be used to measure DNA concentrations (Thermo Scientific, Wilmington, DE, US). After that, the DNA will be kept at -20 °C.

Sequencing of the 16 S ribosomal RNA gene amplicon

The amplification of the 16 S ribosomal RNA gene will be divided into two polymerase chain reaction (PCR) stages. In the first PCR reaction, genomic DNA (gDNA) will be used to amplify the 16 S ribosomal RNA gene's V3-V4 hypervariable region using the primers 341 F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). A reaction mixture comprising of 1X KAPA HiFi Hot Start Ready Mix, 0.1 µL primers 341 F and 805 R, 12.5 ng template DNA, and a total volume of 50 µL per sample will be used to accomplish amplification in 96-well microtiter plates. The following cycling protocol will be used to conduct the reactions in a T100 PCR thermocycle (BIO-RAD): a 3-min denaturation step at 94 °C, followed by 18 cycles of 30 s at 94 °C (denaturing), 55 °C (annealing), and 72 °C (elongation), with a final 5-min extension step at 72 °C. Electrophoresis on 2% agarose gel and ethidium bromide staining will be then used to analyze the amplified products. Following the manufacturer's instructions, amplicons will be measured using the Qubit quantification instrument (Thermo Scientific, Wilmington, DE, US). In the subsequent PCR process, sequencing primers and adaptors will be added to the amplicon products as follows. A reaction mixture made up of 1×KAPA HiFi Hotstart ReadyMix, 0.5 µM fusion forward and 0.5 µM fusion reverse primers, 30 ng Meta-gDNA, and 2 µL of the diluted amplicons will be mixed thoroughly to reach a total volume of 50 µL. The PCR will be carried out using the same cycling protocol, with the exception of cycling number 12. According to the manufacturer's instructions, the amplification products will be purified using Agencourt AMPure XP Beads from Beckman Coulter Genomics in Massachusetts, USA, and quantified as previously mentioned. Equimolar amounts of the amplification products will be combined in one tube. The Qubit quantification system will determine the concentration of the pooled libraries. Illumina MiSeq System will be used for amplified sequencing (Illumina Inc., CA, USA). Illumina Inc.'s MiSeq Reagent Kits v2 will be utilized. Dual-index reads will be used in 2250 bp paired-end sequencing and automated cluster creation.

Preparing samples for a metabolomics analysis

Fecal samples will be analyzed using a targeted metabolomics technique, and a total of 198 compounds will be measured. To reduce degradation, feces samples will be thawed in an ice bath. Each sample will be weighed and then added to a fresh 1.5mL tube, totaling around 10 mg. After that, 25 µL of water will be added, and zirconium oxide beads will be used to homogenize the sample for three minutes. The metabolites will be extracted using 185 µL of ACN/Methanol (8/2). For 20 min, the sample will be centrifuged at 18,000 g. A 96-well plate will be then filled with the supernatant. The subsequent actions will be carried out on a Biomek 4000 workstation (Biomek 4000, Beckman Coulter, Inc., Brea, California, USA). Each well will receive 20 µL of newly prepared derivative reagents. Derivatization will be performed on the plate for 60 min at 30 °C while it is sealed. Then, 350 µL of an ice-cold 50% methanol solution will be added after derivatization to dilute the sample. The plate will be then kept at -20 °C for 20 min before undergoing a 4000 g centrifugation for 30 min at 4 °C. A new 96-well plate with 15 µL internal standards in each well will receive 135 µL of supernatant. Derivatized stock standards will be serially diluted and added to the left wells. For LC-MS analysis, the plate will be finally sealed 565.

Utilizing tandem mass spectrometry and ultra-performance liquid chromatography (UPLC-MS/MS) technology, the microbial metabolite will be measured in the current research (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA). Presented below is a brief explanation of the optimum instrument settings. Both the VanGuard pre-column (2.1×5 mm) and the analytical column (2.1×100 mm) of the ACQUITY UPLC BEH C18 1.7 μM will be used. The sample manager temperature will be 10 °C, while the column temperature will be 40 °C. Mobile phases A and B will be watered with 0.1% formic acid and acetonitrile/IPA, respectively (90:10). The following will be the gradient conditions: At a flow rate of 0.40 mL/min, the time intervals will be demonstrated: 0–1 min (5% B), 1–12 min (5–80% B), 12–15 min (80–95% B), 15–16 min (95–100% B), 16–18 min (100% B), 18–18.1 min (100–5% B), and 18.1–20 min (5% B). The source temperature and desolvation temperature of the mass spectrometer will be 150 °C and 550 °C, respectively, while the capillary will be 1.5 (ESI+) and 2.0 (ESI). The desolvation gas flow rate will be 1000 L/hour.

Statistical analysis

To compare the differences in each RNA or DNA expression between patients with severe and non-severe COVID-19, and controls, we will perform the Kruskal-Wallis test. Additionally, univariate and multivariate logistic regression will be utilized to assess the relationships between each DNA or RNA, as well as the risk of COVID-19 severity, sort of clinical manifestations, and comorbidities. We used G*Power version 3.1.9.7 software for sample size calculation [26]. To achieve a power of 80% and with a type one error of 0.05 and an effect size of 0.25, $N=159$ was calculated. Considering a total dropout rate of 15% a total sample size of 183 participants, categorized into three groups, was calculated.

The control group includes individuals who have tested negative for SARS-CoV-2 through real-time polymerase chain reaction and do not have any underlying medical conditions or are not taking any medications that may influence their gut microbiota. These variables will be included in the final analysis. The patients will be grouped based on their drug history and concomitant medications. To calculate the pack-years of cigarette smoking, the total number of cigarettes smoked per day will be divided by 20, and then multiplied by the total number of years that the individual had been smoking. Also, the Perceived Stress Scale which is a 10-question self-assessment will be used to ask a person to rate the amount of stress they feel with a score of 0–4. During the baseline assessment, the data regarding the participant's diet during the week leading up to their diagnosis will be gathered and entered into dietary analysis software (Diet Plan, Version 6 P3 Forestfield Software, Horsham, UK). Dietary patterns will calculated using five validated scores to measure diet quality [27, 28], diet diversity [29, 30], and compliance with a Mediterranean diet [31].

Ethics and dissemination

Once we plan to recruit the first participant, we will seek appropriate ethics approval. Participation in the study will be voluntary and will require the written informed consent from each participant. Study findings will be disseminated through peer-reviewed publications, conferences, and non-peer reviewed media outlets.

Results

Although it is a research protocol and it has not been initiated, we hypothesize that the gut microbiota composition will be affected by the COVID-19 severity and there might be differences in terms of sex and age of participants. Also, *Faecalibacterium*, *Eubacterium*, and *Bifidobacteria* could be the potential genus that can be reduced in the gut microbiota of severe COVID-19 patients. The changes might explain both the pulmonary and GI presentations of COVID-19.

Discussion

This is a study protocol and no findings of the study are currently available. Nevertheless, we hypothesize that severe COVID-19 can alter the gut microbiota composition at least for several weeks, and there might be sex and age differences in the gut microbiota composition of patients with severe and non-severe COVID-19.

COVID-19 could lead to several organ damage, like respiratory, renal, cardiovascular, neurological, and GI complications [32]. Many efforts have been made since the start of the COVID-19 pandemic to discover a particular therapy for SARS-CoV-2. However, the evidence supporting the effectiveness of the medications currently being used for SARS-CoV-2 is insufficient [33]. In this context, findings of a recent systematic review demonstrated that anti-coronary virus medications have a greater risk of adverse events [34]. Therefore, it is essential to pursue safer and more effective therapies.

Certain cytokines and chemokines such as IL-1β, IL-8, IL-10, tumor necrosis factor-alpha, and interferon-gamma are more abundant in COVID-19 patients than in healthy controls [35]. Additionally, several cytokines may be linked to the severity of the disease [35]. Tao et al. mentioned that alterations in the gut microbiota composition might have a role in the production of inflammatory cytokines in the intestine due to SARS-CoV-2 infection, which may result in the initiation of a cytokine storm [24]. Also, Yeoh et al. highlighted that according to cytokine levels and inflammatory markers in COVID-19 patients, the gut microbiome might play a role in determining the severity of the disease by modulating the

host's immune response [22]. Cheng et al. found that anti-inflammatory butyrate-producing bacteria were depleted and taxa with pro-inflammatory properties were increased in COVID-19 patients during the acute phase compared to non-COVID-19 individuals [36]. Wanglong et al. discovered that *Ruminococcus*, *Blautia*, and *Lactobacillus* genera have a positive association with various host inflammatory cytokines, such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 [37]. The same study found a negative correlation between the inflammatory cytokines indicated above and the genera *Bacteroides*, *Streptococcus*, and *Clostridiales* genera [37]. Furthermore, angiotensin-converting-enzyme 2 expression on GI cells and the connection between the GI and respiratory systems via the gut-lung axis may be the causes of GI symptoms of COVID-19 [38, 39].

Zhang et al. conducted a study that aimed to assess the effectiveness of washed microbiota transplantation in combination with conventional therapy for patients with new SARS-CoV-2 pneumonia, particularly for those who have symptoms of dysbiosis [40]. This study compared the gut microbiome patterns of COVID-19 patients with severe and non-severe diseases, as well as healthy controls. To adhere to new government regulations, NCT04251767 was revoked [40]. The study findings could serve as the foundation for further studies that might produce prognostic indicators and therapy possibilities. Creating recommendations for fecal microbiota transplantation (FMT) as a viable therapy for COVID-19 patients may be helpful. Although FMT is generally regarded as safe and well-tolerated, even in high-risk patients, there are a few short- and long-term treatment-related adverse events that can range in severity from minor to serious [41].

Strengths and limitations

Our study might have some limitations. First, we will include some of the demographic and clinical factors like age, sex, lifestyle (smoking, stress), diet, and comorbidities of participants. However, we might not include some of the potential confounding factors. Therefore, any differences in these factors between healthy individuals and those with COVID-19 should be considered in the final analysis and interpretation of the results. Second, selection and recall biases are probable. So, a cause-effect relationship cannot be concluded from the results. Although we will try to collect and process the samples using standard methods, there is a potential risk of sample contamination.

Despite the limitations, the results of our study could be a backbone for further trials which might lead to the development of prognostic factors and treatment options. Also, it might help develop guidelines for using FMT as a potential treatment for patients with COVID-19, especially with GI symptoms.

Abbreviations

COVID-19	Coronavirus disease 2019
FMT	Fecal microbiota transplantation
gDNA	Genomic DNA
PCR	Polymerase chain reaction
GI	Gastrointestinal
IAA	Iodoacetamide
IL	Interleukin
rCDI	Recurrent clostridium difficile infection
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
TCEP	Tris (2-carboxyethyl) phosphine
TFA	Trifluoroacetic acid

Acknowledgements

None.

Author contributions

SAN and ENM conceptualized the study; SAN, AF, AP, RE, and AK prepared the draft; NR and ENM critically revised it; NR supervised the project. All authors read and approved the final version of the manuscript.

Funding

None.

Data availability

Data sharing is not applicable to this article as no data sets were generated or analyzed during this study.

Declarations

Ethics approval and consent to participate

Ethics approval is not required for the current stage of submission of the protocol.

Consent for publication

Not applicable.

Dissemination to participants and related patient and public communities

Our results will be disseminated through media outlets and presentations at scientific conferences and academic events. Given that no patients were recruited for the study, there are no plans to disseminate the results to study participants.

Competing interests

None declared.

Author details

[1]HIV/STI Surveillance Research Center, WHO Collaborating Center for HIV Surveillance, Institute for Futures Studies in Health, Kerman University of Medical Sciences, Kerman, Iran
[2]Cancer Immunology Project (CIP), Universal Scientific Education and Research Network (USERN), Tehran, Iran
[3]School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4]Gastroenterology & Liver Diseases Research Center, Research Institute for Gastroenterology & Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[6]Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[7]Department of Clinical Biochemistry, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[8]Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[9]Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

[10]Network of Immunity in Infection, Malignancy and Autoimmunity (NIIMA), Universal Scientific Education and Research Network (USERN), Tehran, Iran
[11]Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[12]Research Center for Immunodeficiencies, Children's Medical Center, Tehran University of Medical Sciences, Dr. Qarib St, Keshavarz Blvd, Tehran 14194, Iran

Received: 15 April 2024 / Accepted: 22 May 2024
Published online: 01 June 2024

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