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# Faecal microbiota transplantation (FMT) in Norwegian outpatients with mild to severe myalgic encephalomyelitis/ chronic fatigue syndrome (ME/CFS): protocol for a 12-month randomised double-blind placebo-controlled trial

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## ABSTRACT

**Introduction** The observed alteration of the intestinal microbiota in patients with myalgic encephalomyelitis/ chronic fatigue syndrome (ME/CFS) and the effect of transferring a healthy gut flora from a faecal donor using a faecal microbiota transplantation (FMT) will be explored in this trial.

**Methods and analysis** This is a protocol for a randomised, double-blind, placebo-controlled, parallel-group, single-centre trial, with 12 months follow-up. 80 participants will be included and randomised (1:1:2) to either donor FMT (from two different donors) or placebo (autologous FMT). Participants will be included by the International Clinical Criteria for ME/CFS. The clinical measures of ME/CFS and disease activity include Modified DePaul Questionnaire, Fatigue Severity Scale (FSS), Hospital Anxiety and Depression Scale (HADS), 36-Item Short Form Health Survey (SF-36), ROMA IV criteria, Food Frequency Questionnaire, Repeatable Battery for the Assessment of Neuropsychological Status, heart rate variability testing and reports on the use of antibiotics and food supplements, as well as biobanking of blood, urine and faeces.

The primary endpoint is proportion with treatment success in FSS score in donor versus autologous FMT group 3 months after treatment. Treatment success is defined as an FSS improvement of more than 1.2 points from baseline at 3 months after treatment. Adverse events will be registered throughout the study.

**Ethics and dissemination** The Regional Committee for Medical Research Ethics Northern Norway has approved the study. The study has commenced in May 2019. Findings will be disseminated in international peer-reviewed journal(s), submitted to relevant conferences, and trial participants will be informed via phone calls.

**Trial registration number** NCT03691987.

## STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ In-depth characterisation of participants and biobanking at multiple time points will enable research on possible underlying mechanisms and mediators of an altered intestinal microbiota and might provide a future novel treatment modality.
⇒ Whole-genome sequencing will provide information about both bacterial composition and functional potential of the microbiome in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), complementing primary and secondary endpoints elucidating the causality between an altered microbiota and ME/CFS.
⇒ A study with a different donor chosen by the same criteria, with repeated treatments and/or another delivery method than enema (capsulated faecal microbiota transplantation, endoscopic delivery) might give a different result.
⇒ Patients with very severe ME/CFS are not included in the study.
⇒ Double-blind, randomised, placebo-controlled design.

## INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a chronic, complex, multisystem disease, with a wide spectrum of different symptoms, and no effective cure. Symptoms can be divided into neurological, immune system related, gastrointestinal-related, urogenital and related to autonomic dysfunction. Postexertional malaise is a common complaint, which describes a delayed exacerbation of symptoms following physical or mental activities. There are profound variations in the symptomatology, the severity of the disease and the disease progression.

There are no established or validated biomarkers nor any diagnostic tests to determine the diagnosis. The disease is diagnosed

based on patient-reported symptoms and requires exclusion of other medical conditions. There are different sets of diagnostic criteria. One of the most common is the International Consensus Criteria,[1] applied in this study.

Current treatments do not offer patients suffering from ME/CFS adequate relief of symptoms. ME/CFS profoundly affects the quality of life in patients and little is known about the prognosis. Recovery rates have been reported to be under 10% in adults.[2] All age groups can be affected, but it most commonly occurs between 20–40 years of age. About three-quarters of patients are female. The prevalence of ME/CFS ranges from 0.2% to 2.5%.[3 4]

ME/CFS and irritable bowel syndrome (IBS) coincide with the presence of bowel complaints, fatigue and an altered intestinal microbiota suggested to play a role in the pathophysiology.[5–14] Symptoms very similar to the Rome 4 diagnostic criteria for IBS are subcriteria for diagnosing ME/CFS by the International Consensus Criteria.[1] While 90% of IBS patients experience fatigue as a distressing symptom,[7] an IBS lifetime rate of 90% is found in ME/CFS[9] with more than 50% of ME/CFS patients meeting the IBS diagnostic criteria in several studies.[8] Faecal microbiota transplantation (FMT) is the process of transferring gut flora from a healthy donor to the intestines of a recipient. FMT is in general considered safe when strict donor screening is conducted. Mild and self-limiting side effects such as diarrhoea, constipation, abdominal discomfort, nausea and bloating are reported, but long-term follow-up data are needed.[15 16] Our study group, among others, has found symptom relief of FMT on both bowel complaints[17] and fatigue[18] in IBS,[19] leading to the work on this study testing the efficacy of FMT in ME/CFS.

Thus, there is a knowledge gap and a substantive need for high quality research that identifies mechanisms involved in ME/CFS pathophysiology to develop diagnostic biomarkers and effective treatment strategies. The present protocol aims to obtain an extensive set of biological samples in the process of testing a novel intervention for ME/CFS.

## Rationale

The pathophysiology of ME/CFS is poorly understood. Different hypotheses regarding the aetiology include autoimmunity,[20 21] immune dysregulation,[22] mitochondrial dysfunction,[23 24] genetic predisposition,[25] autonomic dysfunction,[26] neuroendocrine dysregulation,[27] neuroinflammation[28] and altered composition of the gut microbiome.[5]

The microbiota gut brain axis is a bidirectional system with routes for communication that includes the immune system, tryptophan (TRP) metabolism, the vagal nerve and the enteric nervous system, involving microbial metabolites such as short-chain fatty acids (SCFAs), branched chain amino acids and peptidoglycans.[29–33] The diverse symptomatology and pathophysiology of ME/CFS can be caused by disturbances in the communication routes and associated signalling pathways of the microbiota gut brain axis, mediated from an altered gut microbiota.[5 11] A recent study found microbes implicated in TRP, butyrate and propionic acid production that were largely depleted in ME/CFS.[34] Another study found an increased production of SCFAs by microbial fermentation in the gut of ME/CFS patients, which may be associated with deleterious effects on host energy metabolism.[11]

Furthermore, alteration of the gut microbiota interacting with the vagal nerve may constitute a pathological signalling pathway in ME/CFS. Vagal activity can be assessed by the time between each heartbeat, commonly referred to as heart rate variability (HRV). When the autonomic nervous system is in a state of sympathetic overdrive the HRV decreases.[35 36] In ME/CFS a decrease in HRV can predict an increase in fatigue severity and poor sleep quality.[37–39] Moreover, an exaggerated stress response is suggested to increase local gut inflammation and gut epithelial permeability.[35] An altered gut flora accompanied by microbial translocation (indexed by elevated LBP, lipopolysaccharide (LPS), LPS-binding protein (LBP) and soluble cluster of differentiation (sCD14)[5]) and a proinflammatory immune response is found in ME/CFS.[40 41]

FMT is suggested as a strategy to correct the observed alterations and obtain a healthy microbiota, which in turn may cause symptom relief through multiple routes and associated signalling pathways for communication in the microbiota gut brain axis.[42] Moreover, increase in HRV may follow symptom relief after donor FMT and restoration of a healthy gut microbiota, if the vagal nerve is involved in dysfunctional signalling caused by an altered gut microbiota.

A published study on transplantation of enteric bacteria in ME/CFS shows promising results, however, inclusion criteria, evaluation method, lack of control group and poor study design precludes any firm conclusions.[43] To the best of our knowledge, this is the first study to assess the efficacy and safety of FMT in ME/CFS in a randomised controlled trial.

## Treatment cost considerations

From a health economic perspective, FMT for ME/CFS is a promising treatment. ME/CFS is associated with substantial costs to patients, relatives, healthcare systems and society.[44–47] This includes expenses due to work incapacity, productivity losses and early retirement, as well as medical expenses, healthcare and informal care.

## Hypotheses of the study

### The hypotheses of the main study

- Gut microbiota dysbiosis is an important factor in the development of ME/CFS and restoration of a normal gut microbiome can treat the syndrome.
- ME/CFS can be subcategorised on baseline characteristics for prediction of FMT treatment effect.
- Donors can be subcategorised by effect size making it possible to detect patterns and optimise future intervention strategies.

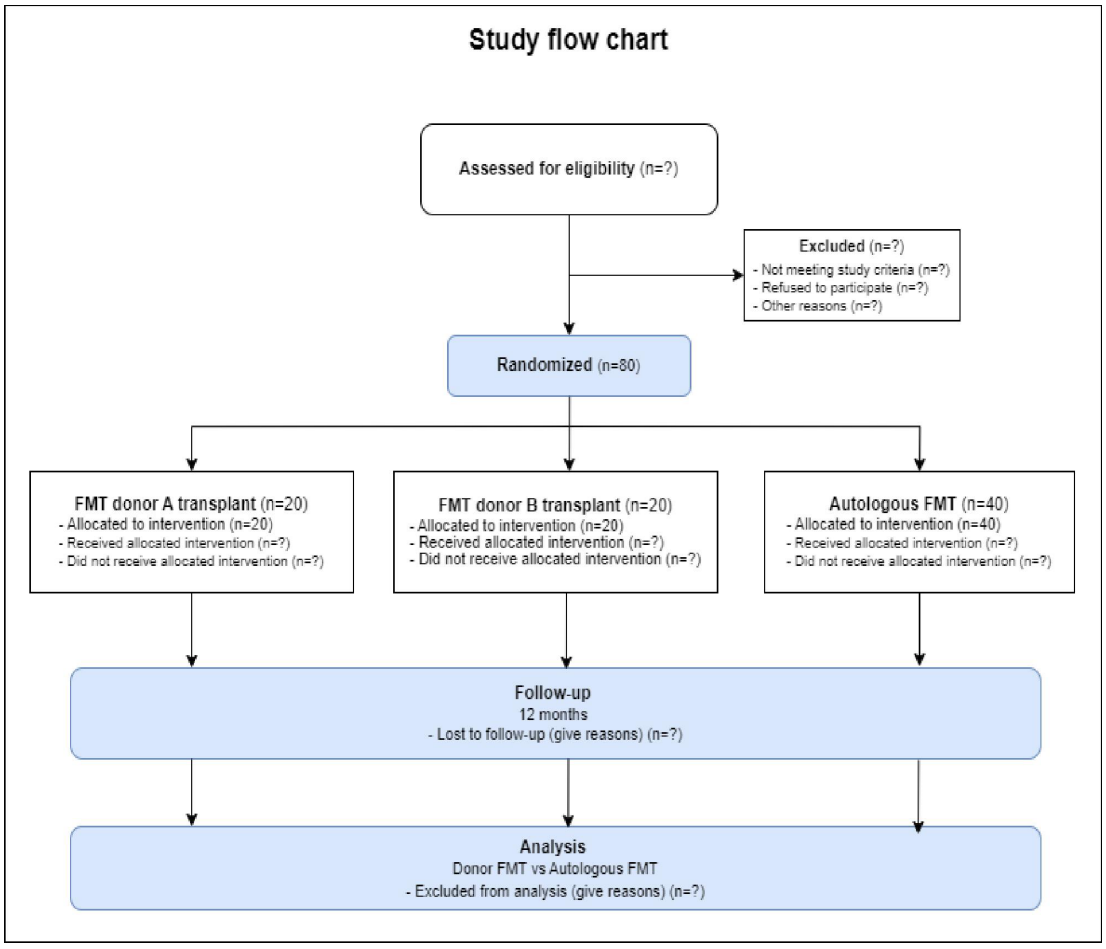


**Figure 1** Study flow chart. FMT, faecal microbiota transplantation.

### Hypothesis of the add-on study

Recovery of a normal gut microbiota by treatment with FMT from a healthy donor restores the equilibrium between the sympathetic and parasympathetic nervous system responsible for maintenance of autonomic homeostasis. Restoration will cause an increase in parasympathetic activity characterised by an increase in vagal activity, indexed by an increase in HRV.

## METHODS AND ANALYSIS

### Trial design

This trial will be performed as a randomised, placebo-controlled, double-blind, single-centre 12-month trial, performed at the University Hospital of North Norway Harstad, Norway. The trial is designed to compare the efficacy of donor FMT and autologous FMT in 80 adult ME/CFS patients. The treatment is randomised in fixed blocks of size four (see figure 1). Primary and secondary endpoints will be analysed with two study arms (donor FMT vs placebo). Outcome assessment will be based on patient-reported outcomes obtained after treatment in the time frame 1–12 months (see figure 2). Faeces, blood and urine are obtained before and at 3 and 12 months after treatment (see figure 2). A stool sample from each donor FMT is obtained to ensure traceability between each donor stool and donor FMT recipient. Analysis of the faecal microbiome in autologous FMT, donors and the corresponding donor FMT recipients will complement the primary and secondary endpoints.

It is optional for participants in the main study to participate in the add-on study testing the effect of FMT on vagal activity, indexed by change in HRV before and at 3 months after treatment with donor or autologous FMT. Only participants included in the clinical study are eligible for inclusion in this add-on study. This feature is not included in the research protocol until 40 patients are included in the main study.

### Participants

A total of 80 patients with ME/CFS will be enrolled. They will be recruited from all parts of Norway, by signing up and giving their contact information to the study personnel. Participants will have to meet the eligibility criteria listed in table 1.

### Faecal donors

Two faecal donors will be recruited from the local area and they will have to meet the eligibility criteria listed in table 1. Before enrolment as a donor, there will be a screening process with an interview, clinical examination and screening of blood, urine and faeces, according to

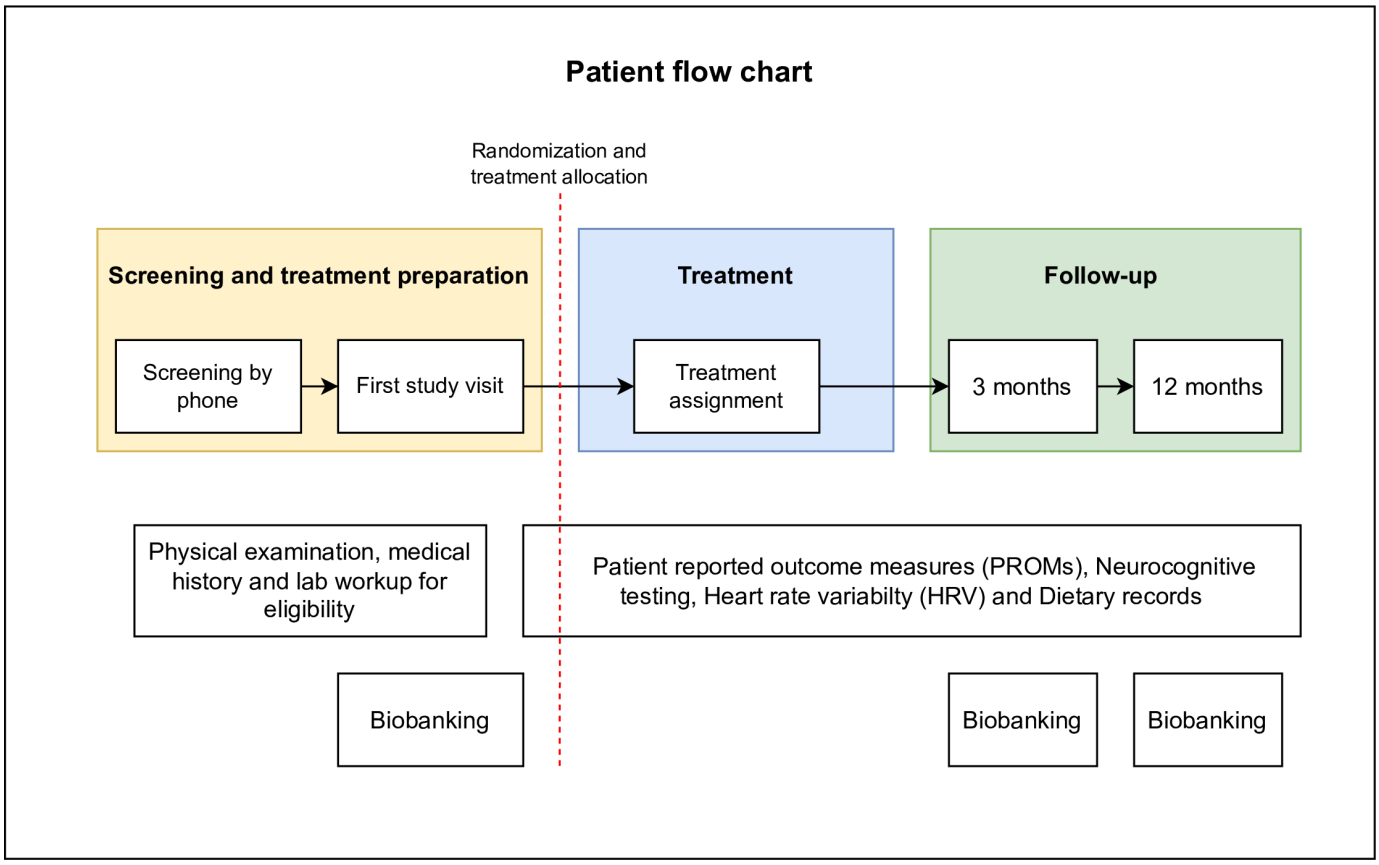


**Figure 2** Patient flow chart.

the European consensus criteria from 2017.[48] In addition, donors are screened for signs of an unhealthy gut microbiota profile, indexed by the Genetic Analysis dysbiosis test.[49]The laboratory screening will be repeated every fourth week during the stool delivery period.

### Healthy controls

Forty healthy controls, matched to the study population in gender and age, will be recruited. Inclusion criteria will be further described in an updated version of the study protocol.

## Interventions

### Preparation of transplants

A placebo FMT from each participant is prepared during the inclusion process. All donor FMTs are prepared before inclusion of the first participant in the study. If donor FMTs are not assigned within 2 years after processing, they will be disposed and replaced with new donor FMTs, preferably from the same donors.

The placebo and donor transplants were prepared as follows:

- 50-80 grams of feces (freshly delivered from donors and thawed from participants)
- mixed with 25 ml 85% glycerol and 120 mL of isotonic saline
- homogenized with a blender (donor samples) or masher (placebo transplants) for 30 seconds
- poured through a 0.5 mm mesh strainer
- transferred to four 60 ml Luerlock syringes
- stored at -80 °C until assigning of treatment

A placebo FMT from each participant is prepared during the inclusion process. All donor FMTs are prepared before inclusion of the first participant in the study. If donor FMTs are not assigned within 2 years after processing, they will be disposed and replaced with new donor FMTs, preferably from the same donors.

### The FMT procedure

Participants receive FMT at an outpatient clinic, with no antibiotics given prior to the intervention. The participants must do a bowel preparation using sodium picosulphate/magnesium citrate (Picoprep, Ferring) before the intervention. The treatment will be administered by enema. The syringes are thawed in a water bath of 37°C for 1 hour before transferring it to an enema bag, adding 240 mL isotonic saline to the bag.

Procedure for administrating FMT by enema[50]: participants lie on his/hers left side on an adjustable examination bench tilted 15° with the head and body down (Trendelenburg). The examiner does a digital examination. The probe in the enema kit is lubricated and inserted into the rectum. To avoid leaking the examiner inflates a rectal balloon attached to the probe. A clamp is removed so that the FMT treatment in the enema bag drains through the kit and into the proximal colon of the patient.

**Table 1** Inclusion and exclusion criteria for participants and FMT donors

| Participant inclusion | Donor inclusion |
|---|---|
| ► International Consensus Criteria for ME/CFS[2]<br>► 18–65 years<br>► Mild-severe ME/CFS<br>► Fatigue Severity Scale of 5.0–7.0<br>► Symptom duration for 2–15 years | ► Healthy<br>► Age 16–30 years |
| **Participant exclusion** | **Donor exclusion** |
| ► Kidney failure<br>► Congestive heart failure<br>► Immunodeficiency or use of immune-suppresive drugs<br>► Other disease that may explain ME/CFS symptoms discovered during diagnostic work up<br>► Use of antibiotics the last 3 months<br>► Use of low dose naltrexone or Isoprinosin<br>► Pregnancy or breast feeding<br>► Serious endogenous depression<br>► Chronic infectious disease (HIV, hepatitis B or C, etc)<br>► Introduction of new food supplements, change in diet or introduction of new medications the last 3 months<br>► Assessed not to be able to follow the instructions for data and sample collection<br>► Very severe ME/CFS (WHO class IV)<br>► Symptom duration of less than 24 months or more than 15 years<br>► History of abdominal surgery, with the exception of appendectomy, cholecystectomy, caesarean section and hysterectomy<br>► Previous treatment with FMT | ► Use of peroral antibiotics past 3 months<br>► Use of topical antibiotics past 2 months<br>► Tattoo or piercing past 6 months<br>► Former imprisonment<br>► History of<br>Chronic diarrhoea<br>Constipation<br>Inflammatory bowel disease<br>Irritable bowel syndrome<br>Colorectal polyps<br>Colorectal cancer<br>Immunosuppression<br>Obesity<br>Metabolic syndrome<br>ME/CFS<br>Psychiatric disorders<br>Other serious autoimmune disease<br>► Close relatives with serious autoimmune disease<br>► High-risk sexual behaviour<br>► Bowel movements that does not correspond to a Bristol Stool Scale type 3 or 4<br>► Journeys abroad the last 6 months to countries high in antibiotic resistance<br>► Use of food supplements, pre, pro or symbiotics past 1 month<br>► Dysbiosis grade 3 or more by the GA-map Dysbiosis Test |

The REDCap software will be used to obtain all questionnaires except from the Food Frequency Questionnaire and the RBANS.
FMT, faecal microbiota transplantation; ME/CFS, myalgic encephalomyelitis/chronic fatigue syndrome; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status.

How to further position the patient so that the FMT is distributed throughout the colon:

1. Participant holds the left side position for 2 min.
2. The participant turns directly to an abdominal position and holds it for 2 min. The head and body should still be tilted down.
3. The patient turns slowly until lying on the right side and holds this position for 2 min. The bench is then tilted the opposite way (anti-Trendelenburg) and the position is held for 2 min.
4. The balloon that prevents leakage is deflated and the probe removed from the rectum.
5. Participant is in right side position with the bench tilted in neutral position for 5–10 min. If the patient feels urge to defecate he/she should immediately be guided to a toilet to avoid soiling.
6. When getting up the patient should go directly from the position lying on the right to a standing position.

After the intervention, participants have no restrictions on activity level and are asked to keep an unchanged diet without introduction of any new food supplements or probiotics in the follow-up period.

### Biobanking

The faecal samples are collected on four containers at home and stored at −20°C until delivery in a frozen condition at the University hospital of North Norway, Harstad and further biobanked and stored at −80°C. Two of four containers are needed for processing of placebo FMT. Blood samples (full blood, plasma and serum) and urine samples are obtained before treatment, and 3 and 12 months after treatment. Samples are stored in a general biobank for dysbiosis-related research (REK North 184045).

### Metagenomic sequencing analysis

Bacterial DNA will be extracted by QIAamp Fast DNA Stool Mini Kit (cat no 51604) using Qiacube automated sample processing (Qiagen, Hilden, Germany). Sequencing is planned with Illumina technology. For in silico analysis our earlier presented pipeline (REF: DOI: 10.1080/19490976.2020.1794263) will form the backbone for further analysis within taxonomic and functional features, exploring changes following FMT and their correlation with clinical effects of the treatment.

### HRV testing

HRV is obtained before and 90 days after treatment. HRV determined by analysis of R-R intervals is a non-invasive method that provides quantitative evaluation of sympathovagal interactions modulating cardiac function. R-R intervals are the time between each heartbeat. R-R intervals in donor FMT and placebo FMT group will be derived from a 15 min continuous ECG recording before and 90 days after the FMT intervention. The device with electrodes for ECG recording (First beat body guard 2 device, First Beat Technologies, Jyväskylä, Finland) is attached to the skin in the morning, following an overnight fast.[51] Intake of water is allowed during the fast. Participants will rest in supine position in a quiet room for 10 min before the ECG recording is executed.

According to the HRV checklist[52]:

1. Breaths per minute (respiration rate) will be described in mean and SD.
2. Artefacts will be corrected by First beat artefact correction method.[53]
3. The equipment of choice in this study is a device which is available for acquisition.
4. The sequence to analyse will be selected from the 10th to the 25th min of the total 30 min recording for all the participants.
5. To ensure standardised conditions during HRV sampling participants are asked to do the recording in the morning, after an overnight fast. During the recording participants must lie down and not engage in any activities.
6. To obtain HRV data, we use the Firstbeat Bodygard 2 (First Beat Technologies) for continuous ECG monitoring and the Lifestyle Assessment software (First Beat Technologies) to analyse the recording. To calculate the HRV variables in the frequency domain short-time Fourier transformation is applied.

### Monitoring

An independent data and safety monitoring board will be set up prior to launching the study. The monitor will arrange meetings during the study and after the end of the study to review data and check the study progress and all adverse events.

### Patient and public involvement

This project is granted by the Norwegian Research Council as a part of a research programme (BEHOV-ME) where a committee consisting of ME/CFS patients, next of kin and the ME/CFS user organisations contributed in identifying the research needs. The funding request for this study, which comprised the study protocol, was approved by the committee. In addition, the project received additional funding from the Norwegian Patient Organisation (Norges ME-forening), and the organisation has published information about the study on their web page, which can be helpful when recruiting participants. A local user representative has assessed the impact of the intervention and time needed to participate in the study. Furthermore, the participants will be informed about the results by phone, and the manuscript with the main findings will be provided by email.

### End of study

The study ends when the last participant's 12-month follow-up visit is completed. In other words, when 80 participants are assigned treatment and have completed their 12-month follow-up or been withdrawn from the trial, or if a trial discontinuation criterion is met.

### Safety board

The safety board consists of PHJ, RG and LS. Monthly telematics meetings will be arranged for update on project progress and any safety issues. If any adverse events are reported the board will arrange a telematic meeting promptly. FMT will be stopped until the board has discussed further measures. Patient-reported adverse events will be documented in a separate questionnaire. In addition to asking for patient reported adverse events at 1, 3 and 12 months post-FMT (table 2), participants can reach one of the investigators at any time by the phone number indicated in the consent form.

## Endpoints and exploratory evaluations

### Primary endpoint

Proportion with treatment success in Fatigue Severity Scale (FSS) score in donor versus autologous FMT group 3 months after treatment. FSS will be scored based on mean of all the scores (range 1–7).

Treatment success is defined as an improvement of more than 1.2 points on the FSS from baseline and until 3 months after treatment.

Neither the European Medicines Agency nor the US Food and Drug Administration recommend existing patient-reported outcomes (PRO) or set of instruments for optimal measurement of fatigue or other symptoms of ME/CFS.[54] Several different primary outcome measurements have been applied in previous randomised controlled trials.[55] In this study, we will use the FSS to determine the efficacy of FMT on fatigue, which is a highly recommended outcome measure by the National Institutes of Health in the USA.[56]

### Primary endpoint, add-on study

Change in HRV in donor FMT versus autologous FMT group derived from the R-R intervals in the resting continuous ECG recordings before and 3 months after treatment. The primary vagal function outcome will be differences in changes from pre–post treatment between groups in high frequency HRV (HF-HRV; in $ms^2$).

### Secondary endpoints

Difference between donor versus autologous FMT group and change in fatigue by the FSS from baseline and until 12 months after treatment.

Difference between the two groups in quality of life by the SF-36 global score from baseline and until 12 months after treatment. The SF-36 questionnaire is recommended

**Table 2** Overview of data collection during the study

| Data collection | Time point | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | −5 weeks | −4 weeks | −1 week | 0 | +1 month | +3 months | +6 months | +9 months | +12 months |
| Written consent | + | | | **Intervention** | | | | | |
| Modified DePaul Questionnaire | + | | | | | | | | |
| RBANS | | + | | | | + | | | |
| Hospital Anxiety Depression Scale | + | | | | | + | | | + |
| Fatigue Severity scale | + | | + | | + | + | + | + | + |
| Heart rate variability | | + | | | | + | | | |
| Food Frequency Questionnaire | | + | | | | + | | | + |
| Quality of life (SF-36) | + | | + | | | + | | | + |
| Patient reported adverse event Q | | | | | + | + | | | + |
| Antibiotics, food supplements Q | + | | + | | | + | + | + | + |
| Roma IV criteria | | + | | | | | | | |
| Disease worsening | | | | | + | + | | | + |
| GI-related items in DePaul Questionnaire | | | | | | | + | | + |
| COVID-19 status | + | | | | | + | | | + |
| Biobanking of faeces | | + | | | | + | | | + |
| Biobanking of full blood, serum and plasma | | + | | | | + | | | + |
| Biobanking of urine | | + | | | | + | | | + |

GI, gastrointestinal; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; SF-36, 36-Item Short Form Health Survey.

as a supplemental questionnaire in research regarding ME/CFS and quality of life, by the National Institute of Neurological Disorders and Stroke (NINDS) in the USA. One study in paediatric ME/CFS recommended an MCID of 10 on the SF-36-PFS.[57] A cross-cultural comparison of SF-36 across 10 countries, including Norway, suggested that the translations are culturally appropriate and the content is equivalent.[58]

Neurocognitive function by the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) total score from baseline and until 3 months after treatment. The RBANS is a concise tool that allows for a brief assessment of a variety of cognitive domains for individuals at the age of 20–89 and age adjusted.[59] RBANS is available in two parallel versions, enabling follow-up testing. In a study in Chinese adults, they estimated the MCID for the total scale score, language, immediate memory, delayed memory, visuospatial/constructional and attention indexes of the RBANS as 8, 9, 10, 10, 6 and 4 points, respectively.[60]

Anxiety and depression by the Hospital Anxiety and Depression Scale (HADS) global scores from baseline to 12 months after treatment.

HADS is a questionnaire designed to screen for mood disorders (with subscales for depression and anxiety) in medical outpatients (non-psychiatric). It is widely used specifically in Europe and Australia but not validated in adult ME/CFS patients. The Norwegian version is considered to be a relatively well-validated screening instrument for psychological symptom burden.[61]

Gastrointestinal-related complaints by the sum score of selected items in the DePaul Questionnaire (29, 30, 46 and 47) from baseline to 12 months after treatment. The DePaul Symptom Questionnaire (DSQ) is recommended as a core instrument for history of disease/injury event in ME/CFS, by NINDS. DSQ was created to evaluate the symptomatology and case definition fulfilment of individuals with ME/CFS.

Frequency of adverse events in the donor versus autologous FMT group from baseline and until 12 months after treatment.

### Exploratory evaluations

Research questions that extend beyond determining the clinical effectiveness of FMT treatment.

Engraftment of donor microbiota at 3 and 12 months.

Taxonomic profile by metagenomic sequencing: Comparison between baseline, post-FMT and donor profile will show if engraftment of donor microbiota parallels clinical response to active FMT.

Change in the intestinal virome at 3 and 12 months and whether recipients virome post-FMT resembles donors' virome.

Change in the nature of host immune and antibody response, and if post-FMT the recipient generates antibody responses to donor microbes.

Change in metabolic pathways of the microbiome in faeces at 3 and 12 months, imputed from metagenomic functional features.

A characterisation of metabolic pathways of the microbiome at follow-up compared with baseline samples in cases with and without treatment effect can indicate pathways that are possibly involved in the pathogenesis of ME/CFS. Moreover, new therapeutic targets or biomarkers may be identified.

Change in the metabolome in faeces, blood and urine at 3 and 12 months.

Combining datasets from metagenomics and metabolomics may help identify key nodes in the metabolic network that can be associated to ME/CFS pathobiology—moreover, in conjunction with the intervention any hypothesis can be tested in silico against the clinical outcomes. Correlating bacterial species to the metabolic profile of blood, urine and faeces before and after FMT treatment in responders and non-responders to FMT treatment may provide causal insights into metabolites responsible to deleterious effects on recipients' energy metabolism.

Change in biomarkers for breach in the gut epithelium (sLPS-binding protein and sCD14) before and after transplantation, at 3 and 12 months.

## Statistical methods and data analyses

### Determination of sample size

The only published open labelled uncontrolled trial with transplantation of enteric bacteria for ME/CFS reported response rate of approximately 58% from one treatment. If the effect is much less, the clinical significance of FMT as treatment modality is questionable due to the nature of the procedure. There are no reviews of the expected placebo effect in this patient group, and validations of questionnaires to assess symptom severity are sparse. From other RCTs performed in this patient group, the response rates to placebo seems to lie somewhere in between 10% and 30%.[62]

Assuming that response rate in the placebo group is either 10%, 20% or 30% and 50% in the treatment group, power analysis (independent proportions in SPSS V.28) yields 20, 39 or 93 participants respectively in each group for a statistical power of 80% in a balanced two-group design ($\alpha$=0.05; 1–$\beta$ = 0.80). As the change from 20% to 50% response rate going from placebo to treatment would represent a clinically relevant difference, and accounting for a couple of dropouts, we, therefore, plan to include and assign the study treatment or placebo to 80 ME/CFS patients in total.

### Randomisation

#### *Allocation sequence generation, blocking and stratification*

A research nurse at the Department of Clinical Research at the University Hospital of North Norway, Harstad (UNN Harstad) creates the allocation sequence using the REDCap software. The treatment is randomised in fixed blocks of size four with two active (one donor A and one donor B) treatments and two placebo; that is,

an allocation ratio of 1:1:2 per block. For the primary endpoint, these will be considered as two study arms, but in some subgroup analyses each unique donor will be considered as one study arm. REDCap creates the blocks and each block will also be stratified on sex. By assigning full blocks with only either male or female participants, to preserve within-block randomness, this also enables continuous recruitment of participants independently of sex.

#### *Allocation: procedure to randomise participants*

Allocation is done in solitude in a closed room with no transparency only containing a freezer with the active transplants (tagged by donor batch ID) and the placebo transplants (tagged by screening number). Before allocation of treatment, a researcher places the FMT-placebos on a table in the room. The allocator can then enter the room as the researcher placing all the placebos leaves the room. The allocator will access the randomisation sequence when entering participants screening number on the REDCap software using a computer in the same room. The allocator will be the only person involved in the study that can access the randomisation programme at the REDCap software. If a screening number is randomised to active treatment, the allocator removes the tag from the placebo transplant and places it on a donor FMT treatment instead. All unused placebo transplants will be disposed immediately. When finished, the allocator places the allocated treatment in a box in a designated freezer. The allocator will build a key file matching the participant's screening number to the donor batch id by updating a key file on paper and store it in a safe not accessible to any others. In addition, the allocator will write the corresponding screening number on tags from the used donor batch and keep them as backup in the same safe. This will allow for tracking of each individual donor batch to a corresponding participant at the end of the trial, when all follow-up is completed.

### Blinding

The study is double-blind. On treatment day, a designated research nurse will collect the allocated treatment from the refrigerator by controlling that the screening number on the allocated treatment match the incoming participants. The designated researcher will be provided with a list of participant names and corresponding screening numbers on treatment days, by author LS. This list will be immediately disposed after treatment. Though blinded, LS will not be present while assigning of treatment.

Participants, investigators and outcome assessors are kept blind to the allocation and intervention. One person will have the designated task of allocating treatment to participants and is kept blind by not knowing the corresponding participant identity to the screening numbers. The only personnel that will have access to the randomisation sequence at the REDCap software is the allocator (UNN, Harstad). The allocator will not have any access to the participants, be involved in inclusion, assigning of treatment, follow-up or data handling at the end of the trial. If any adverse events, PHJ has the authority to emergency unblind, by contacting the department of clinical research at the University Hospital of North Norway, Tromsø. This will be followed by an adverse reaction report. PHJ will decide if it is necessary to unblind participants or the study personnel involved in inclusion, randomisation, allocation, assigning of treatment or follow-up. To be able to maintain blinding in case of adverse events, PHJ will not be involved in any of the former mentioned stages of the trial.

## Statistical analysis plan

We will apply a modified intention-to treat analysis, meaning that we only include participants in statistical analyses that were assigned treatment or placebo. In line with the preliminary sample size calculations, p values less than 0.05 will be considered significant for all statistical tests. All effects and p values will be presented. Whenever available, 95% CIs will be presented alongside the corresponding estimates. Effect size will be reported in OR. The R package 'nlme' and function 'lme' will be used to calculate the linear mixed models, and they will use the restricted maximum likelihood optimisation. The variable denoting unique participants across repeated measurements will be included as a random effect in all the linear mixed models.

### Participant characteristics

The characteristics of the two study arms (ie, active vs placebo) will be described using the following demographic and clinical variables: age, gender, level of education, cohabitation status, employment status, disease duration, body mass index, level of fatigue by FSS, quality of life by SF-36, anxiety and depression by HADS, sleep quality by DePaul, GI-related symptoms by DePaul, level of pain by DePaul, sensitivity to light and sound by DePaul, cognitive impairment by DePaul and RBANS, autonomic symptoms by DePaul and self-reported comorbid psychiatric disease. Categorical variables are reported as frequencies and percentages, and continuous variables using the median and IQR. Neither missing nor imputed values will be used when estimating the study characteristics. However, the number of missing observations per variable and for each measurement will be presented. This will allow retracing of how many observations were used in different linear mixed models, unless otherwise specified; for example, as in the subgroup analyses section.

### Missing data

For statistical methods that cannot handle missing data (ie, other than linear mixed models), missing data will be imputed by using the multiple imputation method fully conditional specification in SPSS V.28 with the number of imputations 'm' set to 5. Due to the heterogeneity in the disease group being studied and because we do not know which variables that will contain missing values, we

consider it too unconstructive and difficult to predefine variables of interest without introducing possible bias in case we may suspect that the data is missing not at random. Rationale will be provided. If there are significant effects in the outlined linear mixed models not dependent on imputed variables, these variables will be added to the imputation as appropriate. Otherwise, verifiable (ie, either through previous studies or calculation of correlations) associations between variables will be used to determine which sets of variables should impute missing values. When imputed data are used, we assume that the data used in a test is complete without missing. The result based on the original data and the 'm' individual imputations will be presented alongside the pooled result which SPSS handles for two sample tests and $\chi^2$ tests.

### Primary analyses

The effect of the two study arms for the categorical outcome (treatment success or failure) will be assessed using a $\chi^2$ test. Effect size will be reported in OR.

### Secondary analyses

The effect of the two study arms for continuous and repeatedly measured outcomes will be assessed accordingly:

Continuous variables, defined by the secondary endpoints, will be evaluated by independent samples t-tests or the non-parametric Mann-Whitney U tests if more suitable.

Measurements repeated for variables, defined by the secondary endpoints, will be evaluated using linear mixed model regression. In the simplest model, categorical treatment group will be included in the model as the only fixed effect. In a potential subsequent model, categorical time of measurement and its interaction with the treatment group will also be included as fixed effects to try and provide more nuanced information about treatment group if treatment group in the simplest model was significant.

### Subgroup analyses

All primary and secondary analyses will be repeated for the different subgroup of donors (ie, A and B) and compared with placebo individually and the other donors, rather than collectively, to determine possible donor efficacy against a baseline or inter donor differences. Other subgroup analyses will include repeating the corresponding secondary analysis for each split on the following variables: comorbid IBS by the Rome IV criteria (yes/no), duration of disease <3 years (yes/no), HRV (dichotomised by median value), HADS anxiety or depression domain score ≥8 (yes/no) and mild ME/CFS severity (yes/no). This will help assess whether any significant effect from the secondary analyses persists (ie, consistency) or any effect changes when the data in turn is limited to each of these subgroups of patients. Regardless of whether the aforementioned subsequent linear mixed model in the secondary analysis is fitted, here this model will only be fitted if the subgroups are sufficiently evenly distributed; that is, no less evenly than approximately 40%/60% or equivalently no less than observations 30.

### Sensitivity analyses

Sensitivity analysis on the primary endpoint will be done where a 0.5 and 0.8 decrease in FSS will be defined as a success, instead of the 1.2 decrease defined for the primary analysis, at 3 months past FMT. Sensitivity analyses will also include linear mixed models to analyse the domains in the questionnaires SF-36 and HADS in separate models. In these linear mixed models, we will follow the same two-step model with a simple model and a subsequent model as in the secondary analyses. For the FSS, we will similarly calculate a linear mixed model for each item in the FSS questionnaire.

### Safety analyses

The time to any first occurring safety outcomes will be described by Kaplan-Meier survival curves grouped by the two study arms. The equality of the survival distribution in those two groups will be tested using the log-rank (Mantel-Haenszel) test. The cumulative proportions of SAEs at the assessment time points will also be described.

### Additional analyses

Other post hoc subgroup analyses and exploratory analyses may also be conducted after data review, which will be appropriately denoted as post hoc. The data sample to be used for the post hoc analyses will also be described.

## ETHICS AND DISSEMINATION

The study protocol with the main study and the add-on study as well as the template consent forms have been reviewed and approved by The Regional Committee for Medical Research Ethics Northern Norway (REK North). Informed consent for the add-on study is obtained in parallel with obtaining the informed consent for the clinical study. Not wanting to participate in the add-on study has no consequence for the participation in the clinical study.

Any modifications to the protocol that may affect the study conduction will be presented to the committee.

Participants may withdraw from the study at any time point, without prejudice, as explained and documented at the time of providing consent.

The study results will be presented to the participants by phone at the end of the study. The results will be submitted to a peer-reviewed journal and presented at national and international conferences, and patient group meetings.

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**Contributors** All authors have made a substantial contribution to the design of this work. PHJ and RG wrote the first study protocol draft. PHJ, LS and RG revised the first draft of the protocol. LS, PHJ and HMH planned and performed

the donor screening programme and were responsible for the implementation of the programme. LS is conducting the clinical examinations. RG, PHJ and LS have written the statistical analysis plan. All authors contributed to the protocol paper, reviewed it critically and approved the final version before submission.

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