Fecal microbiota transplant from healthy lean donors to morbidly obese individuals: effect on insulin resistance and other obesity-related parameters. A randomized controlled trial.

NCT02970877

Date: 10-May-2020

Title: Fecal microbiota transplant from healthy lean donors to morbidly obese individuals: effect on insulin resistance and other obesity-related parameters. A randomized controlled trial.

Protocol Number: 16-5475-A

Date of Protocol: 10 May 2020

University Health Network

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Study Product: Fecal filtrate prepared from feces of healthy lean pre-screened donors

Study Start Date: 01 March 2017

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This protocol has been reviewed and approved by the Principal Investigator:

_____ _____ 10 May 2020__

Johane Allard Date

This study will be conducted in accordance with the protocol,

Good Clinical Practice and any other applicable regulatory requirements, including the archiving of essential documents.

1 MATERIAL AND METHODS

1.1 Study Design

This will be a double-blind, placebo controlled randomized controlled trial. Morbidly obese men and women with IR who are eligible for bariatric surgery but did not perform such procedure will be approached. Those consenting will be randomly assigned to either allogenic FMT (prepared from stool donated by healthy lean donors with BMI <23 kg/m^2, matched for gender with the recipient) or autologous FMT (re-infusion of own collected feces; placebo). We plan to randomize 30 patients. Stool, blood, anthropometric measurements, questionnaires, quality of life, depression and anxiety will be assessed and will be collected before FMT and at 1 and 3 months post FMT. At baseline a physical examination will be performed, and will also be preformed if symptoms are present 3-months post-FMT . OM will be assessed before FMT and 3-months post FMT. Intestinal mucosa biopsies will be collected during colonoscopy for FMT administration as well as samples for safety.

1.2 Outcomes

Main outcome: Insulin resistance (HOMA-IR)

S*econdary outcomes*: weight (absolute number, BMI, % change), appetite score, quality of life, anxiety and depression scores

Other measurements: HbA1c, C-peptide, IM in stools (composition and metagenome), amount of selected groups of microorganisms measured by qPCR, blood lipids, gut hormones, blood pressure, physical examination, food intake (energy and % of energy from fat, carbohydrates, protein, fiber intake (g/d)), physical activity (units/day), environmental questionnaire, stool and serum metabolomics.
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1.3 Study Participants

1.3.1 Patients receiving intervention

Inclusion criteria: Morbidly obese men and women, age 18 years or older, fulfilling the 1991 NIH criteria (BMI >40 kg/m^2 or BMI >35–40 kg/m^2 with other severe weight loss responsive comorbidities) (12, 36), who were referred to the Bariatric Clinic at the Toronto Western Hospital for weight loss surgery, but declining or deferring the surgery, will be included. Will accept also patients fulfilling same criteria above who are approaching us via other streams like MTOP website, cinicaltrials.gov and flyers posted in Hospitals and Universities. Patients have to be insulin resistant, which is defined as having a HOMA-IR value >2.73 (129).

Exclusion criteria: Regular intake of: non-steroidal anti-inflammatory drugs; immune-suppressed subjects for any reason (due to diseases or medications like steroids and other immune-suppressant agents); iron supplements; prebiotics or probiotics from other than food sources, antibiotics, or any experimental drug in the 3 months prior to study entry; type 1 or type 2 diabetes, severe chronic gastrointestinal diseases, previous gastrointestinal surgery modifying the anatomy, smoking; pregnancy or breastfeeding.

Participant withdrawal criteria:

All participants:
Any participant may discontinue from the study at any time:

- At the discretion of the investigator
- At the request of the participant (drop-out)

The reason for discontinuation will be clearly documented in the relevant Case Report Form (CRF).

The following will result in withdrawal of a participant from the study:

- Significant non-compliance with the protocol, based on the investigator's judgment following discussion with the study team and/or a co-investigator
- Any other situation where, in the opinion of the investigator, continued participation would NOT be in the best interest of the participant.

In the event that the participant discontinues the study due to a serious adverse event (SAE), a follow-up contact will be made. This contact will be documented in the source document.

If a participant fails to appear for a follow-up examination, up to three documented phone calls and/or registered mail will be used to locate and recall the participant, if possible.

Specific criteria for FMT recipients: Only one single dose of study product will be administered. Patients will be withdrawn from the study, if the FMT cannot be administered for any reasons. Data of these patients will not be included in the study, except for comparison with the patients who did receive the FMT. These patients will be replaced through additionally randomized patients. There will be no follow-up for patients who did not receive FMT.

1.3.2 Healthy lean stool donors

Inclusion criteria: Men or women 18–45 years old, BMI 18.5-23kg/m^2, not related to or sharing the household with any of the recipients. Donors will be matched by sex with the FMT recipients.

Exclusion criteria:
The following exclusion criteria are standard at the University Health Network Transplant program for all donors:

- History or symptoms suggestive of underlying gastrointestinal disease, such as:
  - Inflammatory bowel disease
  - Gastrointestinal motility disorder
  - Diverticular disease
  - Other chronic symptoms of diarrhea NYD
  - Episode of acute diarrhea in the last 3 months
  - Irritable bowel syndrome
  - Chronic constipation
- Infection or colonization with transmissible agents, including:
  - HIV-1/2
  - Hepatitis A, B and C viruses
  - HTLV-1/2
  - Syphilis
  - VRE, MRSA, Carbapenemase-producing Organisms (CPO), Extended spectrum beta-lactamase-producing (ESBL) organisms
  - *Clostridium difficile*
  - *Salmonella*, *Shigella*, Enterotoxigenic *E. Coli*, *E.coli* 0157-H7 and other Shiga-toxin producing *E. Coli*, *Yersinia, Campylobacter, Aeromonas, Plesiomonas,* and *Vibrio*
  - Enteric viruses (norovirus, rotavirus and adenovirus)
  - Ova and parasites
  - *Helicobacter pylori*

    - Gonorrhea
    - Chlamydia
- History of active malignancy, or any cancer within the last 5 years (excluding basal cell carcinoma of skin).
- Risk factors for prion-related disease, including: family history of Creutzfeld-Jacob Disease, corneal or dural transplant, or receipt of human-derived pituitary growth factor.
- History of high risk for recent acquisition of HIV, Hepatitis B or Hepatitis C, as determined by self-screening using a questionnaire of risk-associated behavior
- History or signs of chronic sexually transmitted infections, such as genital ulcerative diseases, anogenital herpes, anogenital warts, cancroid or syphilitic lesions.
- Immunosuppression, as determined by history or suggestive physical signs. Items on history that indicate immunosuppression include:
    - use of any immunosuppressive medication within last 6 months, including any dose of prednisone
    - chronic underlying condition known to produce immunosuppression (e.g. systemic lupus erythematosus, end-stage renal disease)
- Physical signs suggestive of immunosuppression include:
    - oral thrush
    - lesions of Kaposi sarcoma
    - disseminated lymphadenopathy
- History or physical findings of chronic liver disease or cholestasis
- Evidence of active encephalitis or meningitis
- Evidence of active systemic viral, bacterial or fungal infection, including malaria and tuberculosis
- Receipt of live vaccine in preceding 30 days
- Receipt of blood transfusion from a country other than Canada in preceding 6 months
- History of dementia or degenerative neurological disorders of unknown etiology
- Recent bite from an animal that may have rabies within the past 6 months
- Antibiotic use in the 6 months preceding donation
- Probiotic agent use for medicinal purposes in the 3 months prior to donation
- Use of cholestyramine within 3 months of donation
- Known and current history of blood in stools
- Travel outside Canada/United States in the past 6 months
- Health care workers
- Persons who have recently been hospitalized or discharged from long term care facilitites
- Persons who regularly attened outpatient medical or surgical clinics
- Persons who have recently engaged in medical tourism
- Use of any prescription or nonprescription medications, supplements, vitamins, herbal remedies on a regular basis
- History of COVID-19 in last 3 months
- Symptoms suggestive of COVID-19 or close contact with known or suspected COVID-19 infected person or travel history of any country known to be affected by COVID-19 in last one month

Additional exclusion criteria that apply to all donors for repeat FMT and the donors in our study:

- History, family history in first degree (blood) relatives, or current screening symptoms (as determined by positive MINI, Ham-A or MADRS) of psychiatric illness (including depression, anxiety disorder, post-partum depression, bipolar disorder, schizophrenia)

- History or family history of autoimmune disease in first degree (blood) relatives
- Family history in first degree (blood) relatives of colorectal cancer
- Anaphylactic food and environmental allergies
- Overweight (BMI ≥25 kg/m^2) at any time during lifetime
- Actual BMI lower than 18.5 or higher than 23 kg/m^2
- Waist circumference > 80 cm for women, > 102 cm for men
- Diabetes or "pre-diabetes", defined as HbA1c > 6% according to FMT screening protocol, or HOMA-IR more than 2.73 (129) specifically for this study
- Smoker (cigarette, marijuana, other)
- Use of recreational drugs
- Greater than 2 alcoholic beverages per day on a regular basis

1.4 Intervention

Morbidly obese participants will receive one single dose of fecal filtrate prepared from feces of healthy lean pre-screened donors (allogenic FMT) or prepared from their own feces (autologous FMT) per colonoscopy. Patients will be followed for 3 months post FMT. Patients will be allocated to allogenic or autologous FMT by randomization. As diet can influence the IM composition (131, 132), all patients will receive a brief initial counselling on a healthy diet, where they will be provided with a sheet listing the most important rules for a healthy diet, a copy of Canada's food guide, and an information sheet on a low glycemic index diet from the Canadian Diabetes Association. These are standard tools that Dr. Allard is using in her clinic for patients with non-alcoholic fatty liver disease at the University Health Network.

1.4.1 Stool collection for FMT

Patients and donors will receive a stool collection kit including a collection bowl with a lid, an insulated bag and cooling elements. They will collect one full stool and keep it in the home refrigerator (4°C) for 24 hours (maximum 48 hours) before bringing it to the hospital in the insulated bag with the cooling elements (*see instructions*).

1.4.2 Preparation of fecal filtrate for FMT

Allogenic and autologous FMT will be produced the same way, using either healthy lean donor stool or the recipient's own stool, respectively.

FMT manufacturing

FMT manufacturing will follow actual methodologies used in Dr. Poutanen's laboratory, based on their previous randomized controlled trial (NCT01226992).

FMT delivery

FMT by colonoscopy will be administered on one occasion using FMT derived from 150 g stool.

- Three 50 g frozen FMT, each in 30 mL conical tubes, from the same donor (i.e. a total of 150 g frozen FMT) will be thawed at 2-5°C over 24-48 hours in order to best maintain the integrity of microorganisms.
- Each thawed FMT will be diluted with sterile 0.9N NaCL to make up a total volume of 100 mL per "50g dose" and place into transport container (total volume 300 mL)
- The diluted filtrate will be transported at room temperature to the endoscopy suite in a biosafety specimen transportation bag.

- Once received by the gastroenterology team the filtrate will be administered within 9 hours throughout the colon using a colonoscope.
- Store 2 aliquots of the filtrate in 2ml vials, labelled with the same unique donor identification number, and frozen in the –80°C freezer in the event that further testing is required in the future.
- The colonoscopy procedure and post-procedure observation will otherwise follow standard medical procedures. The FMT by colonoscopy will be administered by a trained gastroenterologist.

The amount of 150 g stool suspended in 300 mL reflects the range of acceptable stool mass and volume in published literature (102). Freezing the stool up to 6 months (increasing to 12 months as soon as data from Dr. Poutanen lab will become available) in Normal Saline containing glycerol as a cryopreservant has been shown not to affect the efficacy against C. difficile or the viability of representative organisms (133).

Patients who are allocated to the allogenic FMT still provide a stool sample (as this is a double-blind study). The laboratory technician who is allocating the groups based on the randomization list will produce an autologous FMT only for those patients who are in this arm. The FMT products for administration will be labelled only with the study ID of the recipient, in order to keep the double-blind design. The aliquots that are kept from the lean healthy donors for future testing are labelled with the donor's study ID. Allogenic FMT will be delivered only after the corresponding donor has passed the second safety screening (same sample as the first one: stools, urine and blood).

1.4.3 Administration of FMT by colonoscopy

For FMT, subjects undergo standard preparation for colonoscopy (*colonoscopy preparation attached*), starting with adjustment of diet 5-7 days prior to colonoscopy and clear fluids only 2 days before the procedure. Two days before the procedure, patients have to take 2 tablets of Dulcolax at 5:00pm. The evening prior to colonoscopy, 4 L polyethylene glycol laxative solution have to be taken to complete bowel cleansing. Colonoscopy will be performed according to medical and safety standards at the University Health Network. Once received by the gastroenterology team the filtrate will be dispensed into six 60ml syringes. Then the fecal filtrate will be administered while the colonoscope is slowly removed. No FMT will be administered in the ileum, to avoid potential infection due to the bacteria from the filtrate to reach the bloodstream through the small solution of continuity due to the biopsy in the ileum. The whole amount (300ml) of filtrate will be administered in the cecum/ascending colon at an infusion rate of about 50ml/min. At the time of infusion, the patients will be in his left lateral recumbence position and will be asked to maintain a right lateral position for at least 1 hour after the procedure to facilitate as much as possible the permanence of the material infused into the proximal portions of the colon.

1.4.4 Rationale for dose and route of administration selected

Even though FMT is increasingly used in the treatment of *C. difficile* infection and other gastrointestinal diseases in clinical practice and research (102, 103), there are currently no standards for the collection of donor feces and preparation of the fecal filtrate. Usually, the donor specimen is homogenized and filtered and either administered directly or frozen and used later (102, 136). Gastrointestinal tubes, colonoscopy, enema, and recently even oral capsules are used to administer FMT (102). In the present trial we will freeze the filtrate before administration via colonoscopy.

We are preparing the fecal filtrate using standard methods developed at Mount Sinai Hospital, which

have been used in a previous Health Canada approved trial on FMT for *C. difficile* infection (NCT01226992, clinicaltrials.gov). The amount of filtrate to be administered was changed from 50 g feces in 500 mL saline to 150 g in 300 mL, as this stool volume is now the standard amount used in our own FMT program at Mount Sinai Hospital. Colonoscopy is a safe way to administer FMT (102). This route was selected, as it allows us to reach more proximal regions of the gut compared to enema. We decided against small intestinal infusion used by Vrieze for their metabolic syndrome study (26), as we are treating morbidly obese individuals that may have large BMI (>40 kg/m^2) and we are concerned with the higher prevalence of gastroesophageal reflux in this population (137), which may increase the risk of aspiration.

Due to the control arm receiving autologous FMT, we are using frozen fecal filtrate. Patients need to prepare for FMT colonoscopy following standard instructions with clear fluids and a laxative the day before the procedure to clean the colon (*see colonoscopy preparation instructions*). The stool for FMT has to be collected before the adjustment of diet and bowel preparation in order to maintain the original IM (stool need to be collected no later than 3 to 4 days prior to colonoscopy). The use of an autologous FMT control group is essential, as the bowel preparation and colonoscopy itself can induce changes to the IM (134, 135). Lean donor stool will be processed the same way as the autologous stool. Freezing stool by adding glycerol as a cryoprotectant has been shown to yield active FMT preparations (136, 138). Costello and colleagues (133) examined the long-term stability of frozen stools for FMT and confirmed that fecal filtrate for treatment of *C. difficile* infection could safely be stored frozen in 10% glycerol for at least 6 months without loss of clinical efficacy or viability in the six bacterial groups tested. In our study, we will store fecal filtrate for a maximum of 3 months before using it for FMT.

We will follow patients for 3 months post FMT. Vrieze et al. found improvements in IR already 6 weeks after FMT, without weight loss (26). We will extend the observation period, as 6 weeks might not have been sufficient to cause significant weight loss. In addition, we want to see, whether FMT leads to sustainable improvements in IR and IM. Patients will be approached by the MTOP Team and will be offered a 3 years follow-up post-FMT (REB# 16-5404-AE, separate consent form).

1.5 Time line of assessments and measurement

Healthy donors: After successful pre-screening over the phone, the potential donors will receive a copy of the consent form by mail or email. This will be discussed with the study coordinator over the phone. If the candidates agrees to participate, they will come to the hospital for a first visit, where screening questionnaires will be administered and screening blood work will be taken. In addition, the MINI semi-structured interview (130), Ham-A, MADRS and CR-RISC questionnaires will be administered. In order to perform an infectious disease screening, also stools and urine will be collected. If the donors pass the screening, they will be provided with a stool collection kit and forms for further assessments: food record, activity log, and environmental questionnaire. The donors will complete these forms during the week preceding their first stool donation. Three to five stool donations per donor will be collected within 2-4 weeks of successful screening (one donation is equal to 150gr of stools). These samples will be used for FMT. In addition, IM and metabolomics will be measured on one additional sample provided on first donation and frozen right after collection. Between each donation, donors have to fill-in a risk self-assessment and a diet screening form. Final screening will be performed two weeks after last donation, to ensure maximum safety of the samples collected.

For MTOP donors, bookend testing will also be done for AMDROs (MRSA, VRE, ESBL and CPO) occurs at a minimum of two-month intervals. Rescreening for other infectious diseases (stool, urine and blood) occurs at least every 6-months. FMT donations provided up to 2 weeks preceding re-screening are released only if re-screening is negative for AROs and all other screened infectious diseases. In addition, while donating stool, FMT donors complete self-screening questionnaires with every donation

for ongoing assessment for their medical health and risk of acquiring infectious diseases. If risk for infection is determined to be present on the questionnaire (e.g. travel to an endemic area for infectious disease), the donor is placed in a washout period prior to being rescreened to re-donate. Diabetes risk (HOMA-IR score), anthropometric measurements and mental health questionnaires are repeated at least every 12 months.

Morbidly obese patients: Patients will be approached if they have attended the information session and signed the psychosocial study consent where they agree to be contacted for future research studies. We will be targeting specifically those patients who decide not to go ahead with the bariatric surgery at this point in time (i.e. no follow-up arranged four weeks after the orientation session). Patients interested in our study calling us on their own initiative will be screened even if not previously evaluated by the Bariatric Clinic at TWH as long as they fulfil all other requirements. After a short phone screening, the patients receive a copy of the consent form, which will be discussed over the phone by the study coordinator. Then they will come to the hospital for the screening visit, where their medical history and medications are recorded and HOMA-IR will be measured to assess eligibility. Patients will be provided with forms for food records, activity log, environmental questionnaire, and a stool collection kit. If the patient lives far or specifically request it, he can have blood works done in an outside lab and kit will be mailed home. In this case medical history and medications are recorded over the phone. Once eligibility is confirmed depending on HOMA-IR measured at screening, patients will be called back for the baseline visit. The following will be assessed: bloodwork, anthropometric measurements, appetite rating, psychological MINI interview, depression, anxiety, and quality of life questionnaires. Then, an FMT appointment will be scheduled and patients will be randomized. Patients will collect stool samples adequate for a single transplant (150gr) before they start the bowel preparation for the colonoscopy. In the week before the stool sample is collected, they will complete the questionnaires provided at baseline. When they bring their stool sample to the hospital, completed forms will be collected, anthropometric measurements will be performed. Baseline measurements will be repeated at 1 and 3 months post FMT, except for quality of life, depression and anxiety scores.. After FMT patients are required to fill-in a symptoms diary to keep track of any AE possibly occurring. In addition, during the colonoscopy performed to administer FMT, mucosal samples will be taken from the rectum, the colon, and the terminal ileum to assess the mucosa-associated microbiome. Blood and colon secretions will be collected to evaluate the baseline immune status of the subject (infections existing prior to FMT) and colon secretions will be also stored for future research.

1.6 Methods

5.6.1 Stool Measurements

Sample collection and processing: Participants receive a stool collection kit, including a plastic collection/storage container, insulated bag, and cooling elements. Patients will collect one stool sample within 24 h before the study appointment (donors) or prior to the 2-day preparation for the colonoscopy (patients). Stools will be placed in the participant's home refrigerator (+4°C) immediately after defecation. Within 24 h (up to 48 h acceptable) the stool will be brought to hospital in the insulated bag with cooling elements, where the stool will be transferred into sterile sample bags and homogenized a stomacher. In the autologous FMT group and in the donors, 150 g of the stool will be processed for FMT as described above. Another stool sample will be collected within 72 hours prior to colonoscopy preparation and stored in patient's freezer. The sample will be stored in our -80°C freezer and then homogenized and separated. Two tubes containing 20 mg each will be stored for stool metabolomics. Three tubes of about 0.1 g stool will be stored for IM measurement, and the rest will be aliquoted in 10-20 mL tubes and stored for potential future analysis. All samples, except for FMT filtrate, will be stored at -80°C until analysis.

Intestinal microbiome (IM): DNA extraction: DNA will be extracted from samples using the PowerSoil® DNA Isolation Kit aiming for 100 mg of sample for stool and the entire sample for plaque (MO BIO Laboratories, Inc., Carlsbad, CA). The extraction protocol will be modified to include a lysozyme digestion step (incubation at 37°C for 30 minutes) (139). DNA concentration and purity will be measured using a Nanodrop Spectrophotometer (Thermo Scientific, Rockford, IL). Extracted DNA will be stored at -20°C until analysis.

Amplification and sequencing: Samples will be processed according to our standard operating procedures and existing pipeline (https://github.com/ggloor/miseq_bin). The workflow will be frozen as a release at the outset of the analysis so that all analyses are conducted using the same protocol and software versions. The current protocol is in brief as follows. DNA samples are amplified with barcoded primers specific for the V4 rRNA gene variable region using the Earth Microbiome Primers modified to have combinatorial inline barcodes (140). Post-polymerase chain reaction (PCR) samples are pooled in equimolar amounts, merged into a single library, and paired-end sequenced on an Illumina MiSeq using the version 3 chemistry. Samples are overlapped, chimeric sequences removed, and operational taxonomic units (OTUs) determined by the USEARCH set of integrated tools (141). These are instantiated in our existing pipeline. As a complementary approach, we will use DADA2, which generates unique sequences corresponding to strain level variation in the input samples (142). In either case, singleton sequences and OTUs that are less than 0.1% abundant in any sample will be discarded, and tables result that contain the number of reads mapping to a particular taxonomic group in each sample.

Statistical analyses for IM will be done using a compositional data analysis paradigm to acknowledge the distortion of the data that arises due to high throughput sequencing (143-148). This approach has the advantage of simultaneously adjusting for read depth differences between samples (144, 148). Exploratory data analysis will be done using compositional biplots (149, 150) that simultaneously display in a semi-quantitative manner the variation in the samples and in the taxonomic groups using an approach similar to that described by Gloor and colleagues (151, 152). Data will be examined and adjusted for any batch effects using an approach taken for microarray and RNA-seq analyses (153).

Differential abundance between groups will be determined using the ALDEx2 package using either the pairwise or glm methods that accounts for both the distortion in the data and the uncertainty of measurement inherent in these samples (144, 151). We will use an effect-size approach (154), rather than use p-values for significance testing, since effect sizes are known to be more reproducible and predictive in multivariate datasets (155). Since collection of whole metagenome sequence or whole meta-transcriptomes would be prohibitively expensive for the entire number of samples, we propose to use 16S rRNA gene profiles to impute metagenome function and pathway abundance tables using the PICRUSt tool (156), and further downstream analyses of function using a compositional approach as done previously (157).

The internal correlation structure of the microbial communities will be examined using the SPIEC-EASI tool that assumes a sparse and highly compositionally-correlated structure (147). Should the sparsity assumption not be met, we will use the phi-statistic developed by Lovell (148) which is useful for non-sparse compositional data.

Functional microbiome analysis: We will determine the full microbial DNA sequence of the subset of 6 patients at 0 and 3 months, using paired-end sequencing on the Illumina HiSeq. This will serve as internal control for the PICRUSt imputed data and allow us to uncover novel functions that are not represented in existing sequenced genomes. We aim to recover 75 million bacterial reads per sample, and assuming 25% human contamination after depleting methylated DNA, we anticipate multiplexing 2 samples per HiSeq lane. Reads will be assessed for microbiome composition using MetaPhlAn2 (158) and a library

encompassing unique genes for the dataset will be produces and processed as for a recent meta-transcriptomic study of vaginal microbial community (157). Reads that are not assigned with this pipeline will be assembled using the Trinity assembly pipeline and assembled fragments will be compared to existing genes to determine if there are any novel functions (159). We have a dedicated 64 core computer with 128 GB of RAM available through SharcNet for this task. Differential abundance of functions and pathways will be examined using the ALDEx2 tool (145, 160) and LEfSe (161) for the imputed data.

Metabolomics analysis: Fecal samples will be collected and stored at -80°C until metabolomics analysis via nuclear magnetic resonance (NMR) spectrometry. Fecal extracts will be prepared by mixing 20 mg of frozen fecal material with 1 mL of saline phosphate buffer in D2O (deuterium oxide), followed by centrifugation (18,000×g, 1 min). Fecal supernatants will be removed and filtered through 0.2 μm membrane filters as described by Le Gall and colleagues (163). The samples will be transferred to a standard NMR tube for ^{1}H-NMR spectral analysis on a 500 MHz Inova spectrometer. Spectral fitting for metabolites will be done using the standard Chenomx 500 MHz metabolite library. Typically 90% of visible peaks are assigned to a compound and more than 90% of the spectral area can be routinely fit using the Chenomx spectral analysis software. These methods have been perfected at the Metabolomic Innovation Centre (164). As for the full microbial DNA sequencing, this measurement will only be performed, if additional funding can be acquired.

1.6.3 Bloodwork

Sample collection and processing: Blood will be drawn in the morning after 12 hours fasting, based on the standard procedures at the University Health Network Laboratory Medicine Program. As insulin secretion is pulsatile, three samples will be taken at 5-min intervals, and the average of the three measurements will be calculated (165). Serum for metabolomics measurements will be collected into tubes without anticoagulant, placed on ice immediately, and centrifuged at 4°C, 2,800×g for 15 min. Serum will be aliquoted and frozen at -80°C until analysis. Samples for TNF-α, IL-6, and vitamin E will be collected in EDTA containing tubes, separated by centrifugation (910 × g, 10 min) and stored at -80°C until analysis. The same will be done for plasma endotoxin, but blood will be collected in pyrogen free heparin tubes (Monovette, Sarstedt, Nuernbrecht, Germany). Samples for hormone analysis will be collected in tubes containing protease inhibitors and other added chemicals described below, then separated by centrifugation (910 x g, 10 min) and stored at -80°C until analysis.

Analyses of standard blood biochemistry: The following standard tests will be performed at the Laboratory Medicine Program at the University Health Network:

Glucose: enzymatic hexokinase method (Bayer Advia 1650 Glucose package)

Insulin: microparticle enzyme immunoassay (MEIA) (Abbott Architect i2000)

Hemoglobin A1c: High-performance liquid chromatography (Variant)

C-peptide: chemiluminometric technology (Immulite 2000)

Plasma lipids: Triglycerides, total and high density lipoprotein (HDL) cholesterol are measured using the Abbot Architect. Low density lipoprotein (LDL) cholesterol is calculated as total cholesterol – HDL cholesterol.

Homeostasis model of assessment (HOMA):

Insulin Resistance HOMA-IR = glucose [mmol/L] x insulin [mU/L] / 22.5 (166, 167). Patients with insulin resistance (HOMA-IR >2.73) (129) and healthy donors with HOMA-IR ≤2.73 are eligible.

Serum metabolomics: Serum will be thawed and ultra-filtered for removal of large molecular weight proteins and lipoproteins. The metabolomics analysis by nuclear magnetic resonance spectroscopy is performed as described above for stool samples.

1.6.4 Nutritional and other assessment

Anthropometry: Weight, BMI, waist circumference and waist to hip ratio are measured. Change in these parameters and excess weight loss (in %) between baseline and 1 month as well as between baseline and 3 months post FMT will also be assessed.

Food intake: Three-day food records will be kept. Participants will eat their regular meals and itemize the food using the 2D Food Portion Visual chart (Nutrition Consulting Enterprises, Framingham, MA) to estimate portion sizes. The data will be analyzed using Food Processor SQL (ESHA Research) to assess the intake of macro- and micronutrients (168, 169) (*food record form enclosed*).

Physical activity: Patients will record their physical activity (daily life activities and sports exercises) for seven days, including time, type and duration and intensity of these activities. Units of exercise will be calculated (1 unit = 30 min mild, 20 min moderate, 10 min strenuous, or 5 min very strenuous activity) (170) (*food/activity forms enclosed*).

Appetite will be assessed in a fasting state, using a validated tool in obese subjects (172, 173). Research participants will be asked to rate their level for each feeling on a 100 mm visual analogue scale, therefore participants will have a numerical ranking for each feeling in mm which can be used for statistical analysis (*see attached form*).

Blood pressure: will be measured in a seated position according to clinical practice.

1.6.5 Psychological assessment

At baseline to all participants and 1 and 3 months after FMT for patients, we will also administer: *The Montgomery-Åsberg Depression Rating Scale (MADRS),* a 10-item questionnaire which psychiatrists use to measure the severity of depressive episodes in patients with mood disorders (174); and *The Hamilton Anxiety Rating Scale (Ham-A)*, a clinician-rated evaluation whose purpose is to analyze the severity of anxiety. The scale is intended for adults, adolescents, and children and should take approximately ten to fifteen minutes to administer (175). Both questionnaires will be administered by the study coordinator.

To identify whether there are particular personality styles that make someone a good donor, we will also administer to the donors at baseline: the *Connor-Davidson Resilience scale (CD-RISC)*, which comprises of 25 items, each rated on a 5-point scale (0-4), with higher scores reflecting greater resilience (176).

Quality of life: This will be assessed using the RAND 36-Item Health Survey 1.0 (RAND SF-36), which has been developed for the Medical Outcomes Study (177). Patients will fill out the form at baseline and 3 months after FMT (*all psychiatric forms enclosed*).

1.7 Statistical Considerations

5.7.1 Sample size and power calculations

Our sample size is determined based on current literature, feasibility and the experience of team members, due to the pilot nature of the proposed study. Following common practice for this kind of studies (e.g. (179, 180)), we plan to recruit until we have randomized 36 eligible patients (18 per FMT

group). With a 20% attrition rate we assume that 15 patients per group will finish the intervention. This represents a greater sample size than the only previous FMT study on metabolic syndrome (26). We are confident that this sample size will allow us to investigate the stated aims.

5.7.2 Statistical analysis plans

A descriptive summary of all measurements will first be compiled using appropriate graphical representations, and means, standard deviations and proportions will be calculated. Baseline characteristics of the randomized groups will be compared to ensure balance on the important variables (e.g. HOMA, BMI). Trajectory plots will be created to examine visually the changes over time.

The changes in the outcome variables of (HOMA-IR, weight, scores for appetite, quality of life, anxiety, and depression) within the two FTM groups (allogenic vs. autologous) will be compared using generalized linear mixed models (GLMM), where the within and between groups changes over time will be examined simultaneously. Factors with known influence on the outcomes will be examined for possible inclusion in our multivariate models as covariates, particularly if found imbalanced between the randomized groups. For patient drop-out, we will check missingness patterns and employ appropriate methods of handling missing data (181). The relationships between changes in outcomes and IM will first be investigated using correlation analysis (Spearman and Pearson correlation coefficients, Chi-square and Fischer exact tests as appropriate), and subsequently using methods for correlated data when considering multiple measures (incl. covariates, multiple time points, etc.). For the latter, we will employ generalized linear and nonlinear mixed models, as well as the generalized estimating equations approach. Goodness-of-fit tests will be applied for model diagnostics (182). In addition, principal component analysis (183), co-inertia analysis will be considered when investigating possible strategies for dimension reduction (where large numbers of variables may be correlated).

Statistical analyses will be performed using SAS 9.4 and R 3.2 (or subsequent versions as they become available), and all tests will be two-sided with a significance level of 5%. Where necessary, p-value adjustments for multiple testing (e.g. Holm's Bonferroni Step-down, Benjamini and Hochberg False Discovery Rate) will be employed (184).

5.7.3 Selection of subjects included in the analysis

As this is a single-dose treatment, we will only include dosed subjects into the analysis. In the initial analysis, all dosed subjects will be included, followed by a per-protocol analysis.

5.8 Randomization and blinding

5.8.1 Randomization list and blinding

Allocation concealment will be maintained by the lab technologist at the UHN-SHS FMT program, who will prepare the fecal filtrates for each patient but will otherwise not be involved in the conduct or analysis of the study. The allocation list will be kept in sequentially numbered envelopes with the group assignments. The group assignments will be based on a blocked randomization list (4 patients per block), created in SAS proc PLAN. The list will be created to randomize 60 patients, even though 36 are planned to be randomized. As we are including only dosed patients in the analysis, this will allow us to randomize additional patients, in case the FMT is not conducted in participants after they were randomized. The list is created by a person at the UHN, who will not be involved in conduct or analysis of the study. The technologist will have to date and sign the opened envelope.

The technologist will open the allocation on the day the patients at providing their baseline stool sample. Only if the patient is allocated to autologous FMT, a fecal filtrate from the patient's own feces is produced. On the day of the FMT, the bags with the fecal filtrate will be provided to the study team

without indication, which type of FMT is provided. Only the patient number will be on the label. Both the patient and the study team (clinical team, study coordinator, data analyst) will be blinded to group assignments.

5.8.2 Unblinding

The code for a patient will be broken, if a serious adverse event occurs that is likely due to the study product, e.g. a new infection of a recipient with a communicable disease, and where the further examination of the incident cannot be delayed until the end of the study based on clinical judgement. The breaking of the code for one patient will not affect the blinding of the other patients during the study. However, it may compromise the blinding during data analysis. In order to maintain the highest blinding level possible, only the study physician with the respective expertise (e.g. Susy Hota and Susan Poutanen for infections) will be unblinded if required for treatment of the patient. The rest of the study team will not be informed.

6 PRODUCT ACCOUNTABILITY

The laboratory technician at Mount Sinai Hospital is responsible for study product accountability, as long as the study is blinded. The technician will maintain current, complete, and accurate records accounting for all study product produced, use of study product by each subject, and product disposition. Records of product dispensed to each subject should include the product lot or batch number, subject number, subject initials, amount dispensed, and date. Records of product disposition should include records of destruction [rendering product unusable by destroying primary packaging (i.e. packaging in direct contact with product)] or return of expired or excess study product. As soon as the study is unblinded, the investigator or designee will take over the responsibility for the product accountability.

7 SAFETY EVALUATIONS AND ADVERSE EVENTS

7.1 Safety Evaluations

Timely and complete reporting of all adverse events (AE) assists in identifying any untoward medical occurrence thereby allowing:

- Continuous safety of study subjects
- A greater understanding of the overall safety profile of FMT.
- Appropriate modification of study protocols
- Improvements in study design or procedures
- Adherence to worldwide regulatory requirements

In this study, all subjects will be assessed according to their outcomes result (IR, HbA1c, weight, appetite). All parameters will be assessed again 1 and 3 months of treatment with FMT. In addition, a symptom diary will be kept for the 3 months to cover solicited and unsolicited AE. Monitoring process and safety assessments will be done by the study coordinator and the sponsor-investigator. The study coordinator will be in direct contact with the patients at each visit. The site investigator will monitor the safety data reported by the study coordinator. The bariatric surgery team will be consulted, in case their expert opinion is needed. As safety assessment, adverse events will be assessed at each visit. Patients may also call the study team at any time in case of concerns.

7.2 Adverse Events

During the study, the site investigator is responsible for the detection and documentation of any adverse event (AE) or serious adverse event (SAE), as defined in this protocol.

7.2.1 Adverse Event Definition

An AE is defined as any untoward medical occurrence in a subject or clinical investigation subject administered a pharmaceutical/medicinal product. An AE does not necessarily have to have a causal relationship with this treatment.

An AE can therefore be any unfavorable and unintended sign (including a clinically significant abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of a medicinal product (investigational or marketed), whether or not considered related to treatment with the medicinal product.

An AE includes:

- An exacerbation of a pre-existing illness, sign, symptom, or clinically significant (as determined by the investigator) laboratory test abnormality
- An illness, sign, symptom, or clinically significant laboratory abnormality that is detected or diagnosed after study drug administration
- Pretreatment or post treatment events that occur as a result of protocol-mandated procedures

An AE does not include:

- The disease or disorder being studied or signs and symptoms associated with the disease or disorder, unless there is worsening of the condition of the disease or disorder

Although an overdose of the study drug or concomitant medication without any clinical sign or symptom is not technically an AE, it should be captured as AE to provide valuable safety data on higher untested doses of study drug.

7.2.2 Procedures for Reporting Adverse Events

Adverse events may be spontaneously reported, obtained through non-leading questioning of a subject. Adverse events will be recorded from the signing of informed consent until the study participant exits the study.

Adverse events do not require expedited reporting. They will be documented on the appropriate case report form/source document. During the duration of the study, the participants will be directed to report any AEs the study team. For more details on documentation and reporting guidelines, see UHN REB Unanticipated Problem Reporting Guidance (*document enclosed*).

At each visit, new AEs are recorded sequentially. The AE term should note the diagnosis whenever possible, not the individual signs or symptoms (e.g., myocardial infarction should be recorded rather than chest pain, and elevated cardiac enzymes). Also recorded are:

- Start and stop date and time
- Whether or not the event is continuing
- Frequency (intermittent, continuous)
- Intensity (mild, moderate, severe)
  - o Mild: usually transient, requiring no special treatment and generally not interfering with usual daily activities
  - o Moderate: usually ameliorated by simple therapeutic maneuvers and impairs usual activities

- Severe: requires vigorous therapeutic intervention and interrupts usual activities. Hospitalization may or may not be required.

- Relationship to study drug (not related, related): identify relationship as "related" if a causal relationship between the investigational product and an AE is at least a reasonable possibility (i.e., the relationship cannot be ruled out).
- Whether or not the AE is serious (i.e., an SAE). If identified as an SAE, the AE should be reported as described below
- Actions taken (none, study drug dose interrupted, study drug discontinued, other medication change, non-drug therapy).
- Outcome (resolved, severity or frequency increased, ongoing, and fatal). An individual AE receives only one outcome.

Adverse events not resolved at the end of treatment will be followed until resolution or until the AE is judged by the investigator to have stabilized.

Laboratory values and clinical findings at the scheduled examinations must be reported as AEs if they:

- Are considered clinically significant by the site investigator,
- Fulfill SAE criteria, and/or
- Cause subject discontinuation from the study.

7.3 Serious Adverse Events

An event that is serious must be recorded on the Serious Adverse Event Report Form. An SAE requires expeditious handling to comply with regulatory requirements. Serious adverse events will be recorded from the signing of informed consent until the participant exits the study.

7.3.1 Serious Adverse Event Definition

An SAE is defined as an AE that meets any of the following criteria:

- Death
- Is life-threatening. A life-threatening AE is any AE that places the subject – in the investigator's opinion – at immediate risk of death from the reaction as it occurred. It does not include a reaction that, had it occurred in a more serious form, might have caused death.
- Persistent or significant disability or incapacity
- Hospitalization or prolongation of existing hospitalization (Hospitalization is defined as inpatient hospitalization for no less than 24 hours and which was not planned prior to the start of the study.)
- Congenital anomaly/birth defect
- Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered an SAE when, based upon appropriate medical judgment, they may jeopardize the subject or may require medical or surgical intervention to prevent one of the other outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room

or at home.

Note: The term “severe” is used to describe the severity of a specific event (e.g., mild, moderate, or severe myocardial infarction); the event itself, however, may be of relatively minor medical significance (such as a severe headache). This is not to be confused with “seriousness,” which is based on the SAE definition that mentioned above. Seriousness (not severity) serves as a guide for defining regulatory reporting obligations.

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