Study Protocol

Version 1.3

Metabolic Outcome of Obese Subjects receiving Fecal Microbiota Transplantation of Lean versus Gastric Bypass Treated Subjects. A Pilot Study.

A randomized, controlled double blinded study

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Confidentiality Statement:

Synopsis

DATE OF PROTOCOL	May 14th, 2021
SHORT TITLE	FMT in Obesity
VERSION	1.3
BACKGROUND	
SPECIFIC AIMS	To assess efficacy and safety of fecal microbiota transplantation (FMT) in patients with morbid obesity. Therefore, we plan this randomized, controlled pilot trial of 30 morbidly obese patients randomized for either FMT from (i) lean volunteers (Lean-FMT intervention groump), from (ii) patients successfully treated with RYGB surgery (RYGB-FMTintervention group), or (iii) for autologous FMT (FMT-placebo group).
NUMBER OF PATIENTS	30 morbidly obese patients, randomized in a 1:1 fashion to either Lean-FMT-intervention, RYGB-FMT-intervention or FMT-placebo. Five lean volunteers and five patients successfully treated with RYGB surgery will serve as donors for FMT in the Lean-FMT or RYGB-FMT intervention group respectively (FMT-intervention donors). Patients in the FMT-placebo group will be treated with autologous FMT.
PATIENT SELECTION	<u>Patients:</u> Consecutive patients from the Endocrinology outpatient clinic suffering from morbid obesity (BMI $\geq$ 40 kg/m^2) will be recruited. <u>Lean-FMT-intervention donors:</u> Normal-weight volunteers with a BMI between 20 and < 25 kg/m^2. <u>RYGB-FMT-intervention donors:</u> Patients followed-up at the Endocrinology outpatient clinic after RYGB surgery with a sustained total weight loss of ≥ 30% at least 12 months after surgery.

Abbreviations

BMI	body mass index
CAP	controlled attenuation parameter
DXA	dual-energy X-ray absorptiometry
EWL	excess weight loss
FMT	fecal microbiota transplantation
FU	follow up
HAV IgG	Hepatitis A virus immunglobulin G
HAV IgM	Hepatitis A virus immunglobulin M
HBc-Ab	Hepatitis B core antibodies
HBs-Ag	Hepatitis B surface antigen
HBs-Ab	Hepatitis B surface antibodies
HCV-Ab	Hepatitis C virus antibodies
HIV-EIA	Human immunodeficiency virus immunoassay
IBS	irritable bowel syndrome
oGTT	oral glucose tolerance test
ScV	Screening visit
SV	Study visit

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1 Background

1.1 Gut Microbiota and Metabolic Disorders

The worldwide burden of obesity and related diseases like type 2 diabetes warrants new therapeutic modalities, as currently available pharmacotherapies and lifestyle modification are incapable of reducing morbidity and mortality rates (1). Surgical interventions, such as the Roux-en-Y gastric bypass (RYGB) procedure, are currently the most effective strategies for the treatment of severe obesity and the only therapeutic option that may enable patients to maintain weight loss in the long-term. One of the most remarkable effects engendered by this procedure in contrast to best medical therapy is its ability to maintain a 20-30% weight loss in the long-term after surgery (2) and its instant improvement in glucose homeostasis (3). However, the surgery is highly invasive, irreversible, and limited as a last therapeutic resort to morbidly obese patients if other treatment approaches have failed.

During the last decade the gut microbiota has emerged as an integral factor that impacts host metabolism and has been suggested to play a vital role in energy control and metabolic homeostasis (4), (5). The gut microbiota regulates energy extraction from otherwise indigestible carbohydrates, determines the integrity if the intestinal epithelial layer, and influences the production and absorption of multiple signaling molecules involved in host metabolism. The gut microbiota is altered in obesity (6), (7), and recent preclinical studies indicate that the gut microbiota may be considered an important environmental factor that together with our genetic predisposition and diet might be related to the progression of obesity and insulin resistance (8), (9). While clinical descriptive studies based on shotgun metagenomics and 16S rRNA enumerations provide critical evidence that the gut microbiota is altered in prediabetes/metabolic syndrome (10) as well as in type 2 diabetes (T2D) (11), (12), and cross-sectional studies in humans have identified even specific microbiota profiles associated with metabolic diseases (13), causation mainly has been demonstrated in animal models. Several animal studies have demonstrated that germ-free mice are resistant to diet-induced obesity, metabolic low-grade inflammation, and glucose intolerance as compared to conventionally raised animals (8), (14), while transplantation of an ‘obese’ microbiota into germ-free mice results in significantly increased adiposity compared to transplantation of ‘lean’ microbiota (15), (14). Intriguingly, also the transfer of microbiota material from RYGB-operated mice to non-operated germ-free mice has recently been shown to result in weight loss and decreased fat mass in recipients (16). Even though these animal studies indicate a causal relation since the metabolic phenotype appears transferable via fecal transplantation, and clinical observational studies (11), (12) have

suggested correlations between altered microbiota composition and metabolism in humans, proof of causality in humans is limited and eagerly awaited.

1.2 Previous Studies Evaluating the Role of Gut Microbiota in Obesity and Diabetes Mellitus

The precise role of gut microbiota in human obesity, metabolism and insulin resistance remains to be elucidated and may pave the way for identification of innovative microbiota-based therapeutics. Fecal microbiota transplantation (FMT) is a relatively old, but still underexplored method, especially in the field of metabolic control that provides a unique opportunity to alter the intestinal microbiota. The first use of FMT in mainstream medicine was described in 1958 for the treatment of pseudomembranous colitis (presumably due to *Clostridium difficile* infection, CDI) by Eiseman et al (17). Recent randomized controlled trials have reported cure rates of 90% or higher in CDI (18), (19) and in general, the treatment effect is lasting and safe, with no related side-effects or newly acquired medical conditions during follow-up, even when performed in vulnerable patient groups (20).

As the burden shifted from infectious to non-communicable disorders, the range of FMT applications extended (21), (22), lately with an increasing focus on the therapeutic potential of microbiome manipulation in reestablishing energy homeostasis and glucose metabolism in clinical obesity and related diseases. Concerning the treatment of metabolic syndrome with FMT, the only two human studies to date were performed by a group from Netherlands, which investigated whether a lean microbiota would improve glucose homeostasis and lipid metabolism in naïve males with metabolic syndrome. Both studies from Nieuwdorp et al suggest that FMT from lean unaffected donors temporarily increases peripheral insulin sensitivity up to a 6-week follow-up (23), (24). The underlying mechanism for this improvement is currently unknown, but butyrate-producing bacteria were significantly increased in both small intestinal biopsies and fecal samples of metabolic syndrome patients treated with lean donor feces (23). Importantly however, the effect declined in all subjects over time with no detectable benefit in peripheral insulin sensitivity or body energy control at 18-week post-FMT follow-up as reported in a subsequent trial from the same group (24). This outcome may be explained by a resilient post-obesity microbiome signature particularly dominating in patients with higher initial fecal microbiota diversity.

1.3 Rationale for the Study

Of note, these clinical outcome data (23), (24) are in line with our own experimental results (yet unpublished data), where we found that FMT from lean donors to conventionalized obese rats resulted in no beneficial outcome on energy or metabolic control. Intriguingly, we observed perfectly different outcome data after transferring microbial material from RYGB-operated rats to obese

animals. Of note, the altered gut physiology following RYGB surgery contributes to dramatic altered microbial ecology (25). Previous studies reported significant top-down effects in the composition and diversity of the metabolic microbiome fingerprint in the short- and long-term after RYGB in humans (26), (27), mice (16) and rats (28), characterized by increased abundance of Proteobacteria such as *Escherichia* and *Enterobacter* in humans (26), (29) and rodents (30), (16). Still most existing studies have a rather descriptive nature and do not allow critical conclusions whether the postoperatively altered microbiome is a bystander of the anatomical gut reconfiguration, a consequence of altered eating behavior, change in adiposity, metabolic control, or whether alterations in microbial composition and/or microbial-derived molecule signaling directly affect the host metabolism towards improved energy and metabolic control.

To address this critical question as to the functional implication of the RYGB-shaped microbiome on surgery outcome and its possible therapeutic potential as a 'knifeless' alternative to beneficially modulate adiposity and related diseases, we performed comprehensive experimental studies. Of note, our yet unpublished animal data clearly demonstrate that the transfer of RYGB microbiota increases levels of butyrate-producing and bile-acid-metabolizing bacteria to counter adiposity and improve systemic insulin sensitivity and lipid metabolism in diet-induced obese (DIO) rats as compared to the transfer of Lean microbiota.

Importantly, data validating these findings in humans are not available, but eagerly awaited. Given the reported findings of a temporarily increased peripheral insulin sensitivity (at 6-week follow-up) after lean FMT of male patients with a metabolic syndrome (23), (24), the aim of the current study is to accomplish a head-to-head comparison of obese patients of both genders treated in a randomized, double-blinded fashion with fecal microbiota from lean donors versus RYGB-operated donors versus autologous fecal transfer with a prolonged follow-up over 24 weeks after FMT.

2 Hypothesis

We hypothesize that RYGB surgery impacts upon the composition and regulatory activity of the microbiota by procedure specific metabolic modulation that in subsequence exerts beneficial effects on the host metabolism. Furthermore we hypothesize that fecal microbiota transplantation (FMT) from obese fecal donors successfully treated with RYGB surgery is superior to FMT from un-operated lean donors to positively affect metabolic outcome parameters in morbidly obese recipients.

To test this hypothesis, we plan this placebo-controlled, double-blinded, randomized pilot trial of 30 obese patients randomized for either (i) allogenic FMT from patients with good treatment response

to RYGB surgery (RYGB-FMT intervention group), (ii) allogenic FMT from healthy un-operated lean donors (Lean-FMT intervention group) or for (iii) autologous FMT (FMT-placebo group).

3 Overview

Patients and stool donors (for RYGB-/Lean-FMT-intervention groups) will be recruited at the Endocrinology outpatient clinic at the University Hospital of Graz. Patients will be randomized in a 1:1:1 manner. In all three study groups, patients will be treated with FMT totaling three times every 7 days after an antibiotic pretreatment.

Patients randomized to the RYGB- FMT-intervention group will be treated with donor stool from previously obese patients successfully treated with RYGB surgery in terms of maintained weight reduction and improved glucose homeostasis. Patients randomized to Lean-FMT-intervention group will be treated with donor stool from un-operated, metabolically healthy and lean individuals, while patients randomized to the FMT-placebo group will be treated with autologous FMT.

For both allogenic FMT interventions, the donor stool from five different patients successfully treated with RYGB surgery (for RYGB-FMT intervention) and from five un-operated, lean and healthy individuals (for Lean-FMT intervention), respectively, will be anaerobically processed before active study period and stored at - 20° C for analysis and subsequent FMT.

In addition, stool from all 30 obese FMT recipients (FMT-intervention groups and FMT-placebo group) will be collected before the active study period, processed anaerobically and frozen at -80° C. Only stool samples from patients randomized to the FMT-placebo group (n=10) will be used as allogenic transplants.

3.1 Primary and Secondary Outcome Measures:

Primary Endpoint:

- Change in insulin sensitivity after treatment (time frame 6 weeks) compared to baseline as assessed by hyperinsulinemic-euglycemic clamp technique.

Secondary Endpoint:

- Change in insulin sensitivity after 16-/24-week treatment compared to baseline as assessed by hyperinsulinemic-euglycemic clamp.
- Change in glucose homeostasis after 6-/16-/24-week treatment compared to baseline as assessed by HOMA-IR model, fasting glucose level, and HbA1C value.

- Change in total body weight, body mass index (BMI) and body composition after 6-/16-/24-week treatment compared to baseline as assessed by Dual-energy X-ray absorptiometry, DXA).
- Change in blood pressure and antihypertensive medication after 6-/16-/24-week treatment compared to baseline.
- Change in fasting lipid profile after 6-/16-/24-week treatment compared to baseline.
- Change in fasting blood liver enzyme levels and liver fat content (assessed by CAP values with the XL probe) after 6-/16-/24-week treatment compared to baseline.
- Change in dietary intake assessed using MyFitnessPal after 6-/16-/24-week treatment compared to baseline.
- Change in metabolic inflammation and endotoxemia as assessed by circulating pro-inflammatory cytokines (TNF-a, IL-6, IL-1ß) and bacterial endotoxins (lipopolysachharide (LPS), LPS-binding protein) after 6-/16-/24-week treatment compared to baseline.
- Change in postprandial release of gut hormones (PYY, GLP-1, GIP, ghrelin, CCK), insulin and bacterial metabolites (SCFA, Bile acids) before (fasting condition) and during a standardized mixed meal test (MMT) (Fresubin 200ml, 400kcal) after 6-/16-/24-week treatment compared to baseline.
- Change in Hunger and Satiety Scores assessed via visual analog scales during the MMT.
- Change in diversity and composition of the fecal microbiota as assessed by 16S rRNA gene profiling after 6-/16-/24-week treatment compared to baseline.
- Change in health-related quality of life and behavior as assessed by established self-report questionnaires after 6-/16-/24-week treatment compared to baseline measuring: (a) eating behavior including trait food craving (FCQ-T-r), hedonic eating (PFS), restrained eating, overeating, and binge eating (EDE-Q), and emotional eating as well as disinhibition (EI); (b) personality factors such as impulsivity (BIS-15) and reward sensitivity (BIS/BAS); (c) mental and physical health, including depression, anxiety, and substance use (PHQ-D), attention-deficit/hyperactivity disorder (ASRS), and quality of life (EQ-5D). All these questionnaires have established reliability and validity.
- Safety and tolerability of repeated FMT assessed by review of adverse event diary card.

4 Study Design and Duration

Estimated Start of study: 06/2021

Estimated End of study: 03/2023

5 Patients and Subjects

5.1 Patient Group

Thirty consecutive patients (male and female) from the Endocrinology outpatient clinic of the University Hospital of Graz suffering from morbid obesity (BMI $\geq$ 40 kg/m^2) will be recruited.

The duration of study participation (active phase) will be about 197 days.

5.1.1 Inclusion Criteria for Patient Group

- Age $\geq$ 18 years
- Morbid obesity defined by a BMI $\geq$ 40 kg/m^2
- Prediabetes or diabetes with HbA1C between $\geq$ 5.7 % OR
- Fasting plasma glucose > 5.6 mmol/l (> 100 mg/dl) (no caloric intake for at least 8 hours) OR
- Random plasma glucose > 11.1 mmol/l (> 200 mg/dl)
- Informed consent

5.1.2 Exclusion Criteria for Patient Group

- Non-Compliance
- Insulin dependent diabetes mellitus, treated with GLP-1 agonists or poorly controlled on oral antidiabetic medications (HbA1C > 8%)
- Use of any weight loss medication or participation in a weight loss program
- History of recent body weight change (defined as body weight loss or body weight gain of $\geq$ 5 kg within the two months preceding study enrolment).
- Use of immunosuppressive medication or immune modulators (glucocorticoids, methotrexate, tacrolimus, cyclosporine, thalidomide, interleukin-10 or -11) within the last three months preceding study enrolment.
- Congenital or acquired immunodeficiencies.
- Anatomical reconstruction of the nutrient passage (i.e. hemicolectomy, resection of small bowel, gastrectomy, sleeve gastrectomy, gastric bypass surgery, biliopancreatic diversion, fundoplication etc) or cholecystectomy.
- Chronic diarrhoea
- History of serious chronic disease including malignancy, rheumatic heart disease, endocarditis, or valvular disease (due to risk of bacteremia)
- Any condition, based on clinical judgment that may make study participation unsafe
- Pregnancy or Breast Feeding

5.2 FMT-Intervention Donors

Five previously morbidly obese patients (male and female) successfully treated with RYGB surgery and a total weight loss of ≥30% kept at least 12 months after surgery (31) will be enrolled as stool donors for the RYGB-FMT intervention group. Donor stool will be collected before active study period at 3 consecutive days and prepared for subsequent transplantation, where each single RYGB-operated donor provides feces for two allogenic FMTs.

In addition, five healthy un-operated lean donors will be enrolled as stool donors for the Lean-FMT intervention group. Lean donor stool will be collected, processed and distributed according to the procedure of the RYGB-operated donor stools.

For all donors the duration of study participation (active phase) will be about 3 days.

5.2.1 Inclusion Criteria for RYGB-FMT Intervention Donors

- Sustained total weight loss of ≥30% ≥12 months after RYGB surgery
- HbA1c < 6.5% without insulin treatment or oral antidiabetic medication
- Age ≥ 18 years
- Informed consent

5.2.2 Exclusion Criteria for RYGB-FMT Intervention Donors

- Intake of pre-, pro- or antibiotics within ≤ 3 months before study entry
- Use of immunosuppressive medication or immune modulators (glucocorticoids, methotrexate, tacrolimus, cyclosporine, thalidomide, interleukin-10 or -11) within the last three months preceding study enrolment.
- Congenital or acquired immunodeficiencies.
- Chronic or acute infectious diseases (specified under 6.2.1)
- Drug abuse
- Anatomical reconstruction of the nutrient passage other than surgical RYGB configuration (i.e. hemicolectomy, resection of small bowel, fundoplication, LSG-to-RYGB transformation etc) or cholecystectomy.
- History of recent body weight change (defined as body weight loss or body weight gain of ≥ 5 kg within the two months preceding study enrolment).
- Chronic diarrhoea or steatorrhea or acute gastrointestinal infection within ≤ 3 months before study entry.

- History of serious chronic disease including malignancy, chronic kidney disease (eGFR < 60 ml/min), heart failure (NYHA ≥ III).
- Any further condition, based on clinical judgment that may disqualify the candidate as an appropriate donor.

5.2.3 Inclusion Criteria for Lean-FMT Intervention Donors

- Normal weight (BMI ≥ 20 to < 25 kg/m^2)
- Age ≥ 18 years
- Informed consent

5.2.4 Exclusion Criteria for Lean-FMT Intervention Donors

- History of overweight or obesity in the past (BMI > 25 kg/m^2)
- History of recent body weight change (defined as body weight loss or body weight gain of ≥ 5 kg within the two months preceding study enrolment).
- HbA1C > 6.5% or treatment with insulin or oral anti-diabetic medication.
- Use of any weight loss medication or participation in a weight loss program
- Use of immunosuppressive medication or immune modulators (glucocorticoids, methotrexate, tacrolimus, cyclosporine, thalidomide, interleukin-10 or -11) within the last three months preceding study enrolment.
- Congenital or acquired immunodeficiencies.
- Chronic or acute infectious diseases (specified under 6.2.1)
- Drug abuse
- Anatomical reconstruction of the nutrient passage (i.e. hemicolectomy, resection of small bowel, fundoplication etc) or cholecystectomy.
- Chronic diarrhoea or acute gastrointestinal infection within ≤ 3 months before study entry.
- History of serious chronic disease including malignancy, chronic kidney disease (eGFR < 60 ml/min), heart failure (NYHA ≥ III).
- Any further condition, based on clinical judgment that may disqualify the candidate as an appropriate donor.

6 Methods

6.1 Schedule of Assessment for the Obese Patient Group

There will be one screening visit (ScV1), a run-in phase, three FMT visits (FMT1, FMT2, FMT3) and three follow-up visits (FU1, FU2, FU3). The screening visit (ScV1), and the three FU visits will consist of two consecutive days. Patients will be managed as outpatients during study participation.

Screening Visit (day -36/35 to day -7/6): Patients will be included at the end of ScV1 if they fulfill all inclusion and no exclusion criterion. Any study-specific procedure will be performed after obtaining informed consent.

At the first day of the screening visit, the following investigations will take place: obtaining informed consent, review of inclusion and exclusion criteria, pregnancy test in female patients (according to BGBI. I Nr. 35/2004), demographics, relevant medical history, current drug intake, physical examination, standard laboratory tests, questionnaires (nutrition intake assessment, Bristol stool scale), stool collection (for anaerobic processing and storage), stool sample collection (2x tubes, one for microbial analyses and one for metabolomics), abdominal ultrasound including measurement of hepatic fat content, and clamp procedure.

At the second day of the screening visit, the following investigations will take place: standardized mixed meal test (MMT), sigmoidoscopy (without bowel preparation), biopsies from colon sigmoideum and rectum (two biopsies from C. sigmoideum, and two biopsies from rectum) and measurement of body composition (lean and fat mass) via dual-energy X-ray absorptiometry (DXA).

Run-in phase (days -3 to day -1): The run-in phase starts three days before the first FMT. For three days (days -3 to -1), patients receive 2 types of antibiotics (Vancomycin & Paromomycin) (32). At day -1, patients have to prepare for colonoscopy with regular bowel lavage.

First FMT visit (day 0): At the first FMT visit, patients will be randomized in a 1:1:1 fashion to either RYGB-FMT intervention, Lean-FMT intervention or autologous FMT-placebo group. Randomization will be performed according to the application 'Randomizer' (www.randomizer.at). After randomization, colonoscopy with biopsies (from C. sigmoideum and rectum) and FMT (250-500ml prepared stool) to the terminal ileum and colon will be performed.

Second FMT visit (day 7 $\pm$ 2): The second FMT visit will take place about 7 days after the first FMT. Here, a sigmoidoscopy with biopsies (2 biopsies C. sigmoideum, 2 biopsies rectum) with subsequent FMT (250-500ml prepared stool) to the left colon will be performed.

Third FMT visit (days 13/14 ± 2): The third FMT will be performed about 7 days after the second FMT and in accordance to the design of the 2nd FMT.

Follow-up visit 1 (days 44/45 ± 5): The first FU visit will consist of two consecutive days and will be performed about 1 month after the last FMT.

At the first day of FU1 visit, the following investigations will be performed: taking medication records, physical examination, standard laboratory tests, nutrient intake (My Fitness Pal), self-report questionnaires as to eating behavior, personality factors and mental and physical health (see secondary outcome measures), stool sample collection for meta-Omics analysis (including 16S rRNA gene profiling, metaproteomics and metabolomics), abdominal ultrasound including quantification of hepatic fat content, and clamp procedure.

At the second day of FU1 visit, the following investigations will be performed: standardized mixed meal test (MMT), sigmoidoscopy (without bowel preparation), biopsies from colon sigmoideum and rectum (two biopsies from C. sigmoideum, two biopsies from rectum) and DXA.

Follow-up visit 2 (days 103/104 ± 5): The second FU visit will consist of two consecutive days and will be performed about 3 months after the last FMT. The second FU visit is designed in analogy to the assessments during the first FU visit as described before.

Follow-up visit 3 (days 197/198 ± 5): The third FU visit will consist of two consecutive days and will be performed about 6 months after the last FMT. The third FU visit is designed in analogy to the assessments during the first and second FU visit as described before.

Visit	**ScV1**		**Run-in**	**FMT1**	**FMT2**	**FMT3**	**FU1**		**FU2**		**FU3**	
Timing	d-36 to -7	d-36 to-7 **+1**	days -5 to -1	d 0	d 7±2	d 13±2	d 44±5	d44±5**+1**	d 103±5	d103±5 **+1**	d 197±5	d 197±5+1
Fasted	X	X	X	X	X	X	X	X	X	X	X	X
Informed consent	X											
Inclusion and exclusion criteria	X											
Randomization				X								
*Pregnancy test**	X											
Demographics/ relevant medical history/ current drug intake	X						X	(X)+	X	(X)+	X	(X)+
Physical examination	X						X	(X)+	X	(X)+	X	(X)+
Standard laboratory tests (Serum for metabolite analysis)	X	(X)+					X	(X)+	X	(X)+	X	(X)+
Questionnaires and assessment of nutrient intake)	X	(X)+					X	(X)+	X	(X)+	X	(X)+
Stool collection (anaerobic processing and storage)d 7)	X	(X)+										
Stool sample collection (for microbial analyses)	X	(X)+		X	X	X	X	(X)+	X	(X)+	X	(X)+
Abdominal ultrasound (incl. CAP)	X	(X)+					X	(X)+	X	(X)+	X	(X)+
Mixed Meal Test		X						X		X		X
Clamp procedure	X						X		X		X	
DXA		X						X		X		X
Antibiosis			X									
Bowel lavage			X (day -1)									
Complete colonoscopy +FMT				X								
Sigmoideoscopy +FMT					X	X						
Sigmoideoscopy		X						X		X		X
Study completion												X

Table 1. Schedule of Assessment. *only females, +if not performed at the first day of visit

6.2 Schedule of Assessment for the FMT-intervention Donors

There will be a screening visit (ScV) and a collection period consisting of three consecutive days. At the screening visit volunteers will be screened for eligibility and stool samples will be collected and evaluated for potential pathogens.

6.2.1 Screening and Assessment of Eligibility for the FMT-intervention Donors

Donor screening for the FMT intervention groups is in concordance with the latest European guidelines from the European FMT working group (Cammarota G, Gut 2016). Accordingly, donors must have a negative anamnesis for intestinal or severe systemic diseases, and a negative medical history for intestinal microbiota modification or severe diseases as specified under 5.2.2 and 5.3.2.Moreover, chronic infectious diseases will be excluded at the screening visit. Testing includes COVID-19 antibodies, hepatitis serology (HAV IgG, IgM, HBs-ag, HBs-Ab, HBc-Ab, HCV-Ab), HIV (EIA), testing for syphilis (VDRL-Test), stool testing for Clostridium difficile toxins A and B, norovirus, stool culture testing for potential pathogenic bacteria (EHEC, salmonella, shigella, yersinien und Clostridium difficile), microscopic stool investigations for worm eggs and parasites.

The written informed consent of the stool donor is obtained by a specialist for internal medicine and has to be obtained before any study-specific procedure.

Medical history	previously obese patients successfully treated with bariatric surgery
	no acute or chronic diseases including neoplasia, severe autoimmune diseases no IBS symptoms, no COVID symptoms
	no infectious diseases
	no antibiosis within 3 months before study inclusion
	no infectious gastroenteritis
	no i.v. drug abuse
	no relatives (1st degree) with colorectal cancer
	no extended abdominal surgery (except for bariatric surgery)
Throat swab	COVID-19 PCR (will be repeated at the stool donation visit)
Serology	HAV-IgM HBV-Ag, anti-HBc Ab HCV-Ab IgG
	COVID-19 antibodies
	HIV Ag and Ab
	Syphilis TPPH
Stool Analysis	Enteropathogenic bacteria (Cl. diff, salmonella, campylobacter, EHEC – Shiga toxin, yersinia, shigella)
	Worm eggs and parasites
	Giardia and cryptosporidia Ab
	Norovirus, Rotavirus
	Calprotectin

Table 2. Donor Screening

6.3 Criteria for Early Study Termination

- Upon request of the patient at any time
- Any condition that, in the opinion of the investigator, could adversely affect the safety of the patient or affect the assessment of the study results
- If patient becomes pregnant or has the wish to become pregnant

If the study terminates early, reasons will be noted in the CRF. Patients will be informed that termination of the intervention does not imply termination of the trial, in general, and study visits will continue as planned.

6.4 Stool Donation and Preparation

FMT-placebo group: In all 30 patients, own stool for potential subsequent autologous transplantation will be collected at Screening Visit 1 and processed anaerobically.

FMT-intervention groups: Stool from five Lean donors (for Lean-FMT intervention group) and from five RYGB-operated donors (for RYGB-FMT intervention group) fulfilling the inclusion criteria according to 5.2.1 and 5.2.3 respectively, will be collected at 3 consecutive days and processed anaerobically for subsequent transplantation.

Stool donors will avoid indigestive foods (e.g. fruit stones, corn, whole-grain products) for at least three days before stool donation. Donors are allowed to drink about half of colonoscopy bowel preparation (e.g. 1 liter of Moviprep®) for stool softening. Stool will be donated in a stool collection cup and should not be older than 6 hours.

Stool from all three donor groups will be stored at -80°C.

6.5 Antibiotics

Antibiosis will be handed to the obese patients at the ScV. Two different antibiotics have to be taken orally from day -3 to day-1 (three days in total) according to the following scheme (32):

- Vancomycin 250 mg 1-1-1-1
- Paromomycin 250 mg 1-1-1

6.6 FMT procedure

After a 3-day antibiotic run-in period, stool will be transplanted three times in an interval of seven days. First FMT will be performed via colonoscopy to the terminal ileum and colon. Here, in total 200ml of stool will be transplanted via 20ml syringes and the working channel of the endoscope.

FMT may be performed in sedation on request of the patient. The 2nd and 3rd FMT will be performed via sigmoidoscopy to the left colon.

6.7 Standard laboratory tests

Blood for evaluation of standard laboratory parameters will be drawn at ScV1, FU1, FU2 and FU 3. In total, 30ml will be drawn according to the following protocol:

tubes	volume	parameters	lab
1 x EDTA	3 ml	blood count	LB 2
1 x Li-Heparin	8 ml	admission profile*	LB 2
1x Citrat	3 ml	Coagulation	LB 2
1x Serum (rot)	8 ml	cytokines, biomarkers	Biobank
Total	**22 ml**		

Table 3. *admission profile: sodium, potassium, calcium, phosphate, creatinine, blood urea nitrogen, uric acid, CK, LDH, ASAT, ALAT, GGT, AP, total bilirubin, total protein, albumin, CRP, LDL, HDL, VLDL, Lp(a), small LDL)

6.8 Specific laboratory tests

Anonymized stool and plasma samples will be sent using dry ice by courier to the University of Leipzig, Germany (Department of Endocrinology, Nephrology and Rheumatology, University Hospital of Leipzig). Plasma samples will be analyzed for the following metabolites: bile acids, SCFA, BCFA, amino acids, biogenic amines, GLP-1, PYY, ghrelin, GIP, leptin, CCK. Stool samples will be used for microbiome analyses.

6.9 Questionnaires

6.9.1 Assessment of Nutrition Intake

A food frequency questionnaire developed by the nutritionists of the Medical University of Graz will be used to assess eating habits. This questionnaire consists of 33 items concerning the intake of carbohydrates, milk products, meat, fish, fruit, vegetable, fat, drinks and snacks. Each item has 6 frequencies (never/rarely, 1-3 times per months, every week, 2-6 times per week, daily, more than once a day) and the patients are asked to tick the most appropriate box.

6.9.2 Self-report Questionnaires

Change in health-related quality of life and behavior will be assessed by established self-report questionnaires at ScV1, FU1, FU2 and FU3. The questionnaires involve aspects of (a) eating behavior

including trait food craving (FCQ-T-r), hedonic eating (PFS), restrained eating, overeating, and binge eating (EDE-Q), and emotional eating as well as disinhibition (EI); (b) personality factors such as impulsivity (BIS-15) and reward sensitivity (BIS/BAS); (c) mental and physical health, including depression, anxiety, and substance use (PHQ-D), attention-deficit/hyperactivity disorder (ASRS), and quality of life (EQ-5D). All these questionnaires have established reliability and validity.

Abdominal ultrasound and measurement of hepatic fat content will be performed at ScV1, FU1, FU2 and FU3.

Hepatic fat content will be measured using a FibroScan device (Echosens, Paris, France) via controlled attenuation parameter (CAP, XL probe). The device estimates liver steatosis in decibel per meter (dB/m). Patients have to be in a fasted state for analysis.

6.10 Mixed Meal Test

For the mixed meal test (MMT) fasted patients will be inserted an intravenous catheter in a distal arm vein after which a baseline (fasted) blood sample will be drawn. After the baseline blood withdrawal, including fasting glucose, insulin, HbA1c, lipids and metabolites (bile acids, SCFA, BCFA, amino acids, biogenic amines, GLP-1, PYY, ghrelin, GIP, leptin, CCK), participants will immediately ingest a standardized palatable mixed meal within 5 minutes (Fresenius Fresubin Energy Drink, 200 ml, 400 kcal per Easy Drink of which the energy content consists out of 35% fat, 45% carbohydrates and 20% proteins). Blood samples will be taken at baseline and at 15, 30, 60, 90, and 120 minutes after ingestion to study the post-prandial metabolism (i.e. glucose, insulin, triglycerides, bile acids, enteroendocrine hormones). All blood samples will be processed and immediately stored at -80°C.

6.11 Hyperinsulinemic Euglycemic Clamp Procedure

To investigate the effect of FMT on glucose homeostasis, we will perform hyperinsulinemic-euglycemic clamps, the gold-standard technique, to determine whole-body and hepatic insulin sensitivity. Of note, clamps will be performed at room temperature at ScV, FU1, FU2, and FU3. After an overnight fast, catheters will be inserted into a distal vein of both arms. One catheter is used for infusion of glucose (glucose 20%) and insulin, while the other is used for sampling of blood.

A priming dose followed by an infusion (40mU/m^2/min) of short-acting human insulin will be applied for 120 minutes. Blood glucose will be clamped at the concentration of 5.5 mmol/l ± 10% (100mg/dl ± 10%) by a variable infusion of 20% glucose. Blood samples for measurement of plasma glucose concentrations will be obtained at 5 minute interval, for measurement of insulin concentrations in 30 minute intervals throughout the clamp.

At the end of each visit, both venous cannulas will be removed. The subjects will rest during the study in supine position, but will have the opportunity to leave the bed during the study if required. During the study visit, patients are allowed to drink water and will receive lunch after end of each visit. Maximum duration of each visit is 4 hours (33).

Steele's single-pool non–steady state equations (34) will be used to calculate rates of glucose appearance (Ra), glucose disposal (Rd), nonoxidative glucose disposal (NOGD; mainly reflecting glycogen synthesis) and endogenous glucose production (EGP), as previously described .

6.12 Dual-Energy X-Ray Absorptiometry (DXA)

Body fat and lean mass will be assessed at ScV1, FU1, FU2 and FU3 using DXA scans (iDXA, GE Lunar, Madison, WI) and lean mass will be calculated as weight (kg) - fat mass (kg).

6.13 Statistics

6.13.1 Sample Size Calculations

The primary aim of this pilot study is to establish clinical protocols, logistics and acquire estimates of expected IS changes in the three groups, all in preparation for a large, multicentre trial.

Data on changes in IS after 6 weeks of FMT can be read off of Fig. 1 from Vrieze et al. and Fig. 2 in Kootte et al. and show that effect sizes are roughly 1 and 0.45 respectively. Assuming an effect size of 0.75 for the pooled intervention groups, then a t-test would have a power of 45% if 10 patients per group are analysed. With a dropout of about 10%, the power is then 41%. With 9 patients analysed per group, the comparison of the two intervention groups would have 12% power to detect an effect size of 0.4 between the intervention arms. The expected width of the confidence interval for the change in IS is less than 4 µmol/kg min.

Once again, this trial is not powered to demonstrate differences between the groups, but to generate rough estimates of them.

6.13.2 Randomization

Randomization: 1:1:1 manner, randomizer.at

6.13.3 Analysis populations

The primary and secondary endpoints will all follow the intent-to-treat (ITT) principle and every randomized patient who provides a valid clamp measurement at baseline will be included in the analyses.

The per protocol (PP) set of patients consists of those who received all planned transplantations and provide valid clamp measurements at baseline and TM3. If the PP set is at least 10% smaller than the ITT set, then an additional PP analysis will be performed for the primary endpoint.

Harms will be listed according to treatment received.

6.13.4 Planned Statistical Methods

Linear mixed models will be used for the majority of endpoints in this repeated measurements setting. In the primary model, the clamp measurements are the dependent variable and time point and group are covariates with patient as a random term. Since changes over time are not expected to be linear, time will not be treated as a continuous variable. Contrasts will be estimated at each time point after baseline and confidence intervals calculated with Tukey's Honest Differences. For the primary endpoint, only the contrast at TM3 will be used and the two intervention groups will be pooled. Corrections for multiple testing at the different time points will not be used considering the pilot nature of the trial. If differences between the pooled intervention and the control group are significant, then the three between group comparisons will be made, without p-value adjustment, because of the closed-testing principle.

Missing data is accounted for within the linear mixed models. As a sensitivity analysis for the primary endpoint, and ANCOVA with the clamp measurement at TM3 as the dependent variable and the baseline value as a covariate will be calculated. Multiple imputation will be used there to deal with missing values.

Tests will be performed on a 5% significance level.

7 Ethical Considerations

An application for this study will be submitted to the Ethics Committee of the Medical University of Graz and once approved, the trial will be registered. Informed consent will be obtained in accordance with the Declaration of Helsinki. All studies will be undertaken after obtaining informed consent. The benefit-risk ratio is favourable.

<u>Study-related blood sampling:</u>

The total volume of blood taken during all study visits is approximately 55 ml in the Screening visit, 160 ml during clamps, and 120 ml during the mixed meal test, respectively.
In addition, one 8ml serum tube will be collected at ScV and at all three FU visits for later analysis of study-related metabolite candidates.

Risks of fecal microbiota transplantation:

FMT has been used for years as a treatment for recurrent *Clostridium difficile* infection (CDI), chronic active inflammatory bowel disease and even in graft versus host disease in clinical and experimental settings. To reduce the risk of transmitting infectious diseases national and international guidelines with recommendations for donor screening had been developed (35, 36). These guidelines were carefully adopted into our protocol and have already been used successfully in previous own studies.
Up to now, more than 400 FMTs have been performed by our working group. Abdominal pain, bloating and increased stool frequency have been reported in the first hours after FMT. Complications such as fever or CRP elevation due to infections are rare, especially when FMT is performed via the lower GI tract. An antibiotic sensitivity test of the donor stool will be performed as part of donor screening. In case of acute, bacterial complications, effective antibiotic treatment is available.
However, in theory there is a possibility to transmit intestinal microbiota susceptible for immunological, metabolic or malignant diseases.
The additional burden and risks of the patient in our study by sigmoidoscopy are very low due to the lack of intestinal lavage. Flexible sigmoidoscopy is a routine procedure in gastroenterological diagnostics with a complication rate of <1: 10,000 procedures. Complications might be perforation and bleeding, the latter being extremely rare in the absence stenosis or anastomosis.

Risks of biopsy

The risk of collecting biopsy samples during endoscopy is minimal. No severe haemorrhage has been reported in studies involving thousands of patients in total (Veitsch AM, Endoscopy 2016). Coagulation testing and bleeding history will routinely be taken prior to examination.

8 Insurance

Participant insurance according to legal requirements will be contracted for patient groups.

9 Budget

A DACH research grant application will be submitted toc… including costs for:

- Study Nurse

10 Persons involved in the Study and their Main Tasks

Study design and critical review of protocol and manuscripts: Julia Mader, Patrizia Kump, Florian Rainer, David Petroff, Julia Münzker, Wiebke Fenske

Development of study protocol: Julia Münzker, David Petroff, Wiebke Fenske, Patrizia Kump, Julia Mader

Recruitment of patients: Julia Mader, Patrizia Kump, Florian Rainer

Data interpretation: Wiebke Fenske, Julia Mader, Patrizia Kump, Julia Münzker

Main responsibilities and authorship for manuscripts and presentations: Wiebke Fenske, Julia Mader, Patrizia Kump, Julia Münzker

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