‘Effect of **In**testinal microbiota tran**s**plantation on **S**a**t**iety and **e**nergy metabolism in female patients with **A**norexia **N**ervosa;

INSTANT-study

(August 2017, version 6.0)

Department of Internal Medicine, AMC-UvA

PROTOCOL TITLE:

''Effect of **In**testinal microbiota tran**s**plantation on **S**a**t**iety and **e**nergy metabolism in female patients with **A**norexia **N**ervosa''; INSTANT-trial

Protocol ID	INSTANT-study
Short title	Intestinal microbiota transplantation in anorexia nervosa
Version	6.0
Date	10-08-2017
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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)**
AE	**Adverse Event**
AR	**Adverse Reaction**
BMI	**Body Mass index**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials**
GCP	**Good Clinical Practice**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IGN	**Gut gluconeogenesis**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
IMT	**Intestinal Microbiota Transplantation**
METC	**Medical research ethics committee (MREC); in Dutch: medisch ethische toetsingscommissie (METC)**
MRI	**Magnetic Resonance Imaging**
PAEE	**Physical activity energy expenditure**
REE	**Resting energy expenditure**
RYBG	**Bariatric surgery**
(S)AE	**(Serious) Adverse Event**
SCFA	**Short Chain Fatty Acid**
SERT	**Serotonin re-uptake transporter**
SPC	**Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)**
SPECT	**Single-photon emission computed tomography**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
T2DM	**Type 2 diabetes mellitus**
Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgegevens)**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**
WHO	**World Health Organisation**

SUMMARY

Rationale: Anorexia nervosa (AN) is a psychiatric disease resulting in severely reduced food intake partly due to altered appetite/satiety balance resulting in very low BMI and the highest mortality rate of all mental illnesses[1]. Several forms of psychological therapies, with or without medication, have been tried, but have not been very successful. The pathophysiology of AN is incompletely understood. It is unclear whether cognitive deficits and an abnormal balance in factors involved in sensing appetite or satiety are the cause or consequence of starvation in AN. In the current study, we want to focus on compensatory changes in satiety and energy metabolism that possibly contribute to the pathophysiology [2]. Preliminary evidence has shown that the gut microbiota might be involved in these compensatory mechanisms, including weight regulation, energy metabolism[3], anxiety and depression[4]. These intestinal microbiota promote colonic serotonin (5-HT) production through stimulatory activities of short chain fatty acids (SCFA) on enterochromaffin (EC) cells [5]. Serotonin pathways are known to contribute to the modulation of a range of behaviours, such as anxiety and is a key regulator of gastrointestinal motility and satiety for food[6 7 8 9 5].One hypothesis is, that an imbalance of the gut microbiota, so called dysbiosis, might cause a dysregulation of serotonin (5-HT) in patients with AN, resulting in anxiety in AN. Indeed, upon examining the composition and diversity of the gut microbiota in patients with AN, significant differences (more gram-negative pathogens) were seen in faeces of patients with AN versus healthy controls[10]. The main question however thus remains if the imbalance of the gut microbiota composition in AN is a cause or a consequence of the disease. Intestinal microbiota transplantation (IMT) can dissect causality from association with respect to gut microbiota and metabolism[11], and perhaps offer an alternative therapeutic approach.
Primary objective: To investigate the effect of intestinal microbiota transplantation (harvest from donors with a normal BMI, 22-25 kg/m2) in patients with anorexia nervosa between baseline and after 6 and 12 weeks. Our primary endpoint is the change in the composition of intestinal microbiota in relation to appetite and satiety and weight. Satiety and appetite will be measured by response to food cues in the CNS using functional magnetic resonance imaging (fMRI)[12] as well as by visual analog scale (VAS), General food cravings questionnaire (G-FCQ), Satiety Labeled Intensity Magnitude (SLIM) scale and plasma markers for satiety and appetite (tryptophan, ghrelin, leptin, neuropeptide Y and orexin levels)[13] upon Nutrient Drink Test (satiety test).
Secondary objectives: The effect of intestinal microbiota transplantation between baseline and after 6 and 12 weeks in: 1. Energy metabolism (as measured by resting energy expenditure (REE), physical activity energy expenditure (PAEE) 2. Serotonin levels, measured by platelet/serum serotonin and 24 hour collected urine (5-HIAA).3. Dietary intake (https://mijn.voedingscentrum.nl/nl/eetmeter) in relation to weight body composition (Body Impedance Analysis, BIA). 4. Psychological response: score on the Yale-Brown-Cornell Eating Disorder Scale (YBC-EDS), obsessive compulsive symptoms and/or compulsivity about feeding will be measured with the Yale Brown Obsessive Compulsive Scale (YBOCS), and assessment of eating behaviour with the Eating Disorder Inventory (EDI-II) and Eating

Disorder Examination Questionnaire (EDEQ). 5. Quality of life (Eating Disorders Quality of Life, EDQOL)

Study design: Double blinded randomized controlled single centre trial.

Study Population: Female patients meeting the DSM-5 criteria for anorexia nervosa, restricting type (307.1; F50.01), BMI <17 kg/m2 and on stable medication and diet.

Intervention: Patients will be randomised into one of the two treatment arms: single allogeneic transplant (female donor with BMI of 22-25 kg/m2)) vs. single autologous (own) transplant by duodenal tube. **Sample Size:** A total of 24 patients (12 patients per treatment arm) are needed plus 12 female donors with a BMI between 22-25 kg/m2.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: Total study duration is 12 weeks (18 hours), during which subjects will visit the AMC 4 times, screening not included, and in total 200 ml blood will be taken (screening 20ml and 60ml at 0,6 and 12 weeks). Day -1 and week 6 and 12: resting energy expenditure, body impedance analysis and mixed meal test will be performed. Day 0 (Baseline): Placement of a duodenal tube by Cortrak (30min), followed by bowel lavage using Klean-Prep, after which intestinal microbiota transplantation will be performed. Placement of Coretrak is a very frequently performed intervention at our department of gastroenterology clinic with a very low (<0.1%) complication rate. The total dose equivalent of the participating patients (aged 18-40 years) will be 0.7mSv for the abdominal X-ray during Coretrak (which is less than the currently allowed 10.0 mSv WHO category IIb).

No side effects of intestinal microbiota transplantation studies are expected (FATLOSE-1 MEC, 07/114; FATLOSE-2 MEC 11/023; FEBALIGO MEC 2013_090, RALSTONIA trial ,MEC 2015/014). Because a strict screening protocol is applied to intestinal microbiota donors at the AMC, the risk of spreading potential pathogens during intestinal microbiota transplantation seems negligible and no long term effects have been reported at our clinic since 2008 (>300 intestinal microbiota transplantations performed).This intervention might benefit patients with AN by improving metabolism and satiety and reduce anxiety. Furthermore, this research will provide valuable therapeutic insight into which gut microbiota affects (serotonin driven) human metabolism, which will help us in developing improved methods to combat AN.

1. INTRODUCTION AND RATIONALE

Anorexia nervosa (AN) is a complex and severe psychiatric disorder that mainly affects adolescent girls and young women and is associated with increased morbidity and mortality[14] [15]. It is characterized by a distorted body image and excessive dieting that leads to severe weight loss (BMI below the 10th BMI percentile) with a pathological fear of becoming obese[16]. The lifetime prevalence of AN is estimated between 0.9% to 4.3%[17] and has the highest mortality rate of any other mental illness[1]. It is estimated that 10% of people with AN die within 10 years of the onset of disorder [18]. Current treatment includes cognitive behaviour therapy and if needed anxiolytic therapy and very rarely forced feeding via a gastroduodenal tube[19]. Despite this treatment regimen, the results are largely unsuccessful and relapse is frequent.

It is unclear whether cognitive deficits and an abnormal balance in factors involved in sensing appetite or satiety are the cause or consequence of starvation in AN.

Besides cognitive deficits, it is hypothesized that compensatory changes in appetite and energy homeostasis are one of the drivers of the underlying pathophysiology and maintenance of underweight in AN[2]. So far it is not completely understood how this regulation between food intake and energy expenditure is orchestrated and what the relative contribution of the central nervous system and the intestine are in relation to weight regulation. A new player in this field of research might be our indwelling bacterial species: the gut microbiota. The gut microbiota in humans consist of more than 100 trillion microorganisms. The environment, including short-term dietary patterns, exerts profound influence on the gut microbiota[6]. Recent animal studies have shown that the gut microbiota are involved in the regulation of appetite and energy expenditure by affecting hormones including incretins and ghrelin that influence metabolic function and areas in the brain associated with eating behavior[20]. This so-called microbiota-gut-brain axis represents a bi-directional signaling axis that regulates bodyweight through balancing appetite, storage and expenditure of energy[21].

The gut microbiota of obese individuals are found to be more effective at extracting calories from food and stimulating host accumulation of fat than microbiotas of lean individuals. For example, faecal samples transplanted to germ-free mice from obese adult humans transmit obesity-associated phenotypes via the gut microbiota[22]. Compelling evidence that the gut microbiota regulates key features of AN, including weight regulation, energy metabolism[3], anxiety and depression[4], provides a strong rationale for exploring the role of this complex microbial community in AN. Based on the existing literature, it is logical

to posit that changes in the gut microbial communities associated with extreme weight loss may perpetuate and contribute to AN via direct effects on weight and mood. Kleiman et. al and Morita et. al both found a dysbiosis (more gram negative Archaea pathogens) in the faecal composition of gut microbiota in patients with AN, supporting the hypothesis that microbial changes might be associated with malnutrition, anxiety and depression in AN (figure 1)[10].

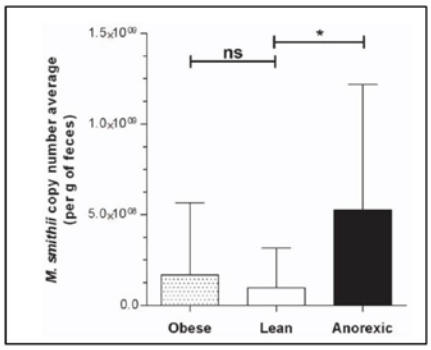

Figure 1. Quantification of the archaeon M. smithii species[3].

This increasing evidence suggesting an interaction between the gut microbiota and the central nervous system (CNS), is recognized as the gut-brain axis[10]. Manipulation of the gut microbiota could hence have a direct effect on neurotransmitter receptors in the CNS. For example, Bravo et al investigated healthy human volunteers who consumed a mixture of probiotics, exhibiting reduced anxiety and depression in comparison to placebo[23]. They highlight the important role of bacteria in the bidirectional communication of the gut-brain axis and suggest that certain organisms may prove to be useful therapeutic adjuncts in stress-related disorders such as anxiety[10]. Moreover Savignac et al. also showed a reduced stress-related behaviour in mice after infusion of Bifidobacteria[24] thus underscoring the therapeutic potential of intestinal bacteria on anxiety and stress.

Another important player in the pathophysiology of AN is thought to be a dysregulation of serotonin (5-HT) [25 26]. Serotonin pathways are known to contribute to the modulation of a range of behaviours commonly seen in individuals with AN. It has been implicated physiological traits such as anxiety, depression, but also satiety for food consumption[27]. Alterations of these circuits may induce obsessive behaviour, anxiety and fear.
The vast majority of serotonin in the human body is produced by enterochromaffin (EC) cells of the gut, where it is synthesized by the rate-limiting enzyme tryptophan hydroxylase (Tph/TPH) 1, and stored in secretory granule prior to release[28]. Reigstad et al. showed that human- and mouse-derived gut microbiota promote colonic Tph1 expression and serotonin production through stimulatory activities of Short Chain Fatty Acids (SCFAs) on EC cells. Since the small intestine is the largest production site of serotonin, it has been suggested that the intestinal microbiota might play a key role in the regulation of AN, including satiety,

gastrointestinal motility, weight maintenance, energy metabolism, anxiety, and depression [6 7 10 8 9 4 29].

The intestinal microbiota transplantation (IMT) is a technique often applied at AMC for an increasing number of diseases[11], and has provided us with a tool to dissect causality from association with respect to gut microbiota and metabolism[11]. In our first pilot study, stool samples from lean male donors were transferred to obese insulin resistant metabolic syndrome patients which resulted in a significantly increased insulin sensitivity and gut microbial diversity[30]. This was associated with a distinct increase in butyrate (SCFA)-producing bacteria, similar to results in other studies[31,32]. Moreover, we found a significant decrease in (VAS list) reported appetite in subjects who were treated with lean donor material as compared to autologous (own) material (see figure 2 below).

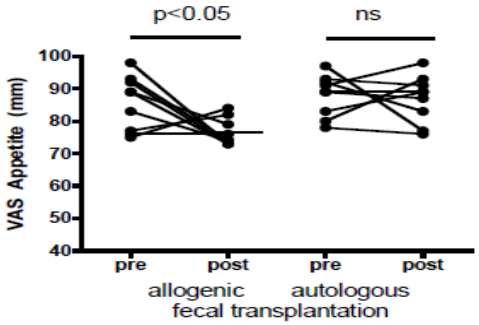

Figure 2. VAS (visual appetite score) showing significant differences in satiety upon allogenic and autologous intestinal microbiota transplantation in male patients with metabolic syndrome.

These findings suggest that the change in intestinal microbiota may play an important role in appetite and potentially weight control. This is supported by a case report showing weight gain in a lean subject that received obese donor microbiota transplant for Clostridium difficile infection[33], suggesting a direct interaction between the gut microbiota and increased appetite and energy harvest from the intestine.
In summary, intestinal microbiota transplantation is a safe and feasible approach that holds promise to prove causality and might comprise a significant therapeutic advance in the treatment of AN [11]. In this trial, we aim to study the therapeutic effects of intestinal microbiota transplantation from healthy female donors with BMI between 22-25 kg/m2 on satiety, metabolism and weight in female subjects with AN.

2. OBJECTIVES

In this randomized controlled trial we aim to investigate the effect of intestinal microbiota transplantation (harvest from donors with a BMI of 22-25 kg/m2) on satiety and energy metabolism in female patients with restricting type anorexia nervosa.

Primary objective: To investigate the effect of intestinal microbiota transplantation (harvest from donors with a BMI between 22-25 kg/m2) in patients with restrictive type anorexia nervosa at baseline,6 and 12 weeks. The primary endpoint is the change in the composition of intestinal microbiota from stool samples in relation to appetite and weight. Satiety and appetite will be measured by response to food cues in the CNS using functional magnetic resonance imaging (fMRI)[12] as well as by visual analog scale (VAS), General food cravings questionnaire (G-FCQ), Satiety Labeled Intensity Magnitude (SLIM) scale and plasma markers for satiety and appetite (tryptophan, ghrelin, leptin, neuropeptide Y and orexin levels)[13] upon mixed meal test. Finally a Nutrient drink test is performed prior to the intestinal microbiota transplantation to measure satiety.

Secondary objectives:
The effect of intestinal microbiota transplantation between baseline and after 6 and 12 weeks on:

1. Energy metabolism (as measured by resting energy expenditure (REE), physical activity energy expenditure (PAEE)
2. Serotonin levels, measured by platelet/serum serotonin and 24 hour collected urine (5-HIAA).

3. Dietary intake (https://mijn.voedingscentrum.nl/nl/eetmeter) in relation to weight body composition (Body Impedance Analysis, BIA).
4. Psychological response: Score on the Yale-Brown-Cornell Eating Disorder Scale (YBC-EDS) and Yale Brown Obsessive Compulsive Scale (YBOCS)and assessment of eating behaviour with the Eating Disorder Inventory (EDI-II) and the Eating Disorder Examination Questionnaire (EDEQ).
5. Quality of life (Eating Disorders Quality of Life, EDQOL)

3. STUDY DESIGN

As an intestinal microbiota transplantation is accompanied by a substantial placebo effect[12], we designed a randomised double-blinded placebo controlled interventional trial. We aim to include 24 patients with anorexia nervosa.

3.1 Screening and inclusion (1 hour)

A screening visit will be performed in the AMC. During this screening in- and exclusion criteria will be verified and, after oral and written explanation of the study, informed consent will be obtained. Medical history will be recorded and physical examination will take place including body weight, height, waist and hip circumference and blood pressure.
A psychiatristwill perform the psychiatric screening to confirm the diagnosis anorexia nervosa, restricting type (DSM-5 307.1; F50.01) as well as the absence of psychiatric comorbidities (mentioned as exclusion criteria on the standardized screening form).
Following the screening visit subjects will keep a diet journal via https://mijn.voedingscentrum.nl/nl/eetmeter until the end of the study. Furthermore, subjects will have fasted overnight before every visit.

If participants are found to be eligible for the study they will be randomised to the following 2 treatment arms:

1. Autologous intestinal microbiota transplantation (n=12)
2. Allogenic intestinal microbiota transplantation from obese subjects (n=12)

3.2 Study visits and measurements

Visit 1: Day -1 (4 hours)

After an overnight fast, subjects will bring 24 hours collected urine and morning stool samples. Then questionnaires (15 min) and fMRI (30 min) , will be conducted followed by resting energy expenditure (REE) of 30 min and body impedance analysis (BIA) will be assessed. After the REE, a 120 min meal satiety test will be performed.
For 7 days prior to the first (day -1) and third visit (week 4) an accelerometer will be worn, to measure physical activity energy expenditure (PAEE).

Visit 2: Baseline: Intestinal microbiota transplantation (5 hours)

Intestinal microbiota transplantation will be performed by placement of duodenal tube (using Coretrak). Thereafter, abdominal X-ray will be performed to check correct placement of duodenal tube. If the tube is placed correctly, bowel lavage by using Klean-Prep is performed (4 hours). After the colonic lavage, the intestinal microbiota is transfused (with autologous or allogenic material according to the randomisation).

Visit 3: 6 weeks (4 hours)

After an overnight fast, subjects will bring 24 hours collected urine and morning stool samples. Then questionnaires (15 min) and fMRI (30 min) , will be conducted followed by resting energy expenditure (REE) of 30 min and body impedance analysis (BIA) will be assessed. After the REE, a 120 min meal satiety test will be performed again.
For 7 days prior to the first (day -1) and third visit (week 4) an accelerometer will be worn, to measure physical activity energy expenditure (PAEE).

Visit 4: 12 weeks (4 hours)
After an overnight fast, subjects will bring 24 hours collected urine and morning stool samples. Then questionnaires (15 min) and fMRI will be conducted followed by resting energy expenditure (REE) of 30 min and body impedance analysis (BIA) will be assessed. After the REE, a 120 min meal satiety test will be performed. For 7 days prior to the first (day -1) and third visit (week 4) an accelerometer will be worn, to measure physical activity energy expenditure (PAEE).

3.3 Overview of study procedures

Week	Screen	-1	-1	-1	-1	0	3	3	3	3	6	7	7	7	7	12
Day		1	3	6	7	1	1	3	6	7	1	1	3	6	7	1
AMC visit no.	1				2	3					4					5
Duration visit in hours	1				3	5					3					3
Fasting					x	x					x					x
Physical examination	x				x						x					x
Blood sample in ml	20				60						60					60
Accelerometer (PAEE)		start			stop		start			stop		start			stop	
Online diet list	start					stop	start			stop		start				stop
X-ray						x					x					x
48 h stool [a]				X	X				X	X						
24h urine sample					x					x				x	x	
Morning stool					x						x					x
VAS, SLIM and G-FCQ [b]					x						x					x
Questionnaires [c]					x						x					x
fMRI					x						x					x
REE [d]					x						x					X
BIA [e]					x						x					x
Satiety meal test					x						x					x
Intestinal microbiota transplantation						x										

a) Stool collection during 2 days (48h) for calorimetry
b) Visual Analog Scale (VAS), Satiety Labeled Intensity Magnitude (SLIM) and General food cravings questionnaire (G-FCQ)
c) Quality of life (Eating Disorders Quality of Life, EDQOL), Yale Brown Obsessive Compulsive Scale (YBOCS), the Yale-Brown-Cornell Eating Disorder Scale (YBC-EDS) and assessment of eating behaviour with the Eating Disorder Inventory (EDI-II) and the Eating Disorder Examination Questionnaire (EDEQ).
d) Resting Energy Expenditure (REE)
e) Body Impedance Analysis (BIA)

4. STUDY POPULATION

a. Subjects

4.1.1 Inclusion criteria

In order to be eligible to participate in this study, patients must meet all of the following criteria:

- Caucasian female
- Older than 18 years
- BMI <17 kg/m^2
- Stable medication use
- Meeting the DSM-5 criteria for anorexia nervosa (ICD-9-CM code 307.1), restricting type (ICD-10-CM code F50.01) . In summary, patients are adhering to caloric restriction relative to requirements, leading to below normal body weight (BMI <18 kg/m^2), and have an intense fear of gaining weight, as well as a disturbed own-body experience and persistent lack of insight in the severity of their condition [16]. Restricting type: during the last three months, the person has not engaged in binge-eating or purging behaviour (i.e. self-induced vomiting or the misuse of laxatives, diuretics, or enemas). Therefore the weight loss is accomplished primarily through dieting, fasting, and/or excessive exercise.

- Subjects should be able and willing to give informed consent

4.1.2 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Medication use, including PPI and antibiotics in last 3 months
- Smoking, XTC, amphetamine or cocaine abuse
- Alcohol abuse (>3/day)
- A concomitant severe psychiatric disorder, as judged by a psychiatrist using the standardized INSTANT study psychiatric screening form (document K6), that renders participants unsuitable for participation in the study.
- Participation in a research protocol involving radiation exposure in the last 2 years.
- Contraindication MRI (pregnancy, pacemaker and metals contraindicated for MRI).
- Cholecystectomy
- Expected prolonged compromised immunity (due to recent cytotoxic chemotherapy or HIV infection with a CD4 count < 240)
- Treatable underlying cause of anorexia/underweight

b. Donors

4.2.1 Inclusion criteria

In order to be eligible to participate as an intestinal microbiota donor in this study, a subject must meet all of the following criteria:

- Healthy Caucasian female

- Older than 18 years
- BMI between 22-25 kg/m^2

4.2.2 Exclusion criteria

A potential donor who meets any of the following criteria will be excluded from participating as donor in this study:

- Use of any medication including PPI and antibiotics
- Diarrhoea
- Cholecystectomy
- HIV, HAV, HBV, HCV, active CMV, active EBV, IBD
- Unsafe sex practice (questionnaire)
- Presence of faecal bacterial pathogens (Salmonella, Shigella, Campylobacter, Yersinia) or parasites
- Positive C. difficile stool test

Individuals with an increased risk for one of the above conditions (homosexual contacts, recent blood transfusions) will be excluded, and donors are not recruited amongst health care providers.

c. Sample size calculation

We based our sample size calculation on a previous study on fMRI in anorexia patients showing that AN subjects that recovered from anorexia had significantly increased activation of posterior cingulate gyrus area (responsible for hunger and satiety drive) during food anticipation (9536 mm3) compared to active AN (1984 mm3)[34]. Assuming that IMT can induce 50% increase in activated posterior cingulate gyrus area at 6 weeks compared to baseline (from 1984 to 2976 mm3) with SD 1000m3, whereas own feces has no effect or a reduction (from 1984 to 1684 mm3).Thus, with 80% power and alpha of 0.05 (two sided T-test) the sample size is 22. Taking a 10% dropout into account, we will include 24 subjects.

5. NON-INVESTIGATIONAL PRODUCT

Not applicable.

6. METHODS

6.1. Study parameters/endpoints

6.1.1. Main study parameter/endpoint

- Intestinal microbiota composition (morning stool samples)
- Response to food pictures. The primary outcome is the difference in BOLD signal, i.e. in CNS activation, in predefined regions in the CNS in response to viewing food pictures.
- Satiety and Appetite, measured by visual analog scale (VAS), General food cravings questionnaire (G-FCQ) and Satiety Labeled Intensity Magnitude (SLIM) scale and plasma markers for satiety and appetite (tryptophan, ghrelin, leptin, neuropeptide Y and orexin levels) upon Nutrient Drink Test

6.1.2. Secondary study parameters/endpoints

- Energy metabolism, measured by resting energy expenditure (REE) and physical activity energy expenditure (PAEE)
- Serotonin levels (platelet/serum serotonin and 24 hour collected urine for 5-HIAA.
- Dietary intake (https://mijn.voedingscentrum.nl/nl/eetmeter), weight and body composition (Body Impedance Analysis, BIA).
- Psychological response: score on the Yale-Brown-Cornell Eating Disorder Scale (YBC-EDS), obsessive compulsive symptoms and/or compulsivity about feeding will be measured with the Yale Brown Obsessive Compulsive Scale (YBOCS), and assessment of eating behaviour with the Eating Disorder Inventory (EDI-II) and the Eating Disorder Examination Questionnaire (EDEQ).
- Quality of life (Eating Disorders Quality of Life, EDQOL)

6.2. Randomisation, blinding and treatment allocation

Patients will be randomised by computer programme (ALEA). Blinding will be guaranteed by combined intestinal microbiota transplantation in both groups. On the day of transplantation, both patient and donor will deliver morning stool samples. Randomisation and preparation of the transplant material will be performed by one of the research assistants. He/she is the only person who will know which treatment the patient will be given and will have no further part in the study. The samples will be put in a 500ml glass bottle and will look like a brownish fluid not recognizable as stools from the donor or patient and given to the investigator who will perform nasoduodenal tube infusion. Patient, donor and investigator will all be blinded. Ample experience with this procedure (> 300 intestinal microbiota transplants at the AMC in the last 8 years) has been obtained.

6.3. Study procedures

Screening of donors

Potential donors will be thoroughly screened according to the protocol used in the FECAL & FATLOSE trial as previously approved by our METC (MEC 07/114 and 11/023). Donor material will be collected from donors who are screened according to the guide. Please also see 4.2.2 exclusion criteria (for donors).

Intestinal microbiota transplantation

Preparation of transplant material

Donor will deliver a fresh stool sample (150-250 grams on average) on the day of infusion (produced within 6 hours before use). After collection in a plastic flask, stool will be covered by saline and stored at room temperature. Time of collection will be written down. All procedures will be performed at the department of Clinical Bacteriology, AMC. All steps are performed in fume-hood by an experienced lab co-worker or the investigator. Fresh stool solution will be mixed during 10 minutes until fully homogenised. Hereafter, the stool solution is poured through a clean metal sieve and food derived debris of large size will be removed. This step will be repeated. Hereafter, the homogenised solution will be decanted through a clean metal funnel into a 1000ml sterile glass bottle. Thereafter, a sample of the processed infusate will be taken for later analysis and the bottle will be kept on normal room temperature (17ºC) until the patient is finished with bowel lavage.

Bowel lavage

Colonic lavage will be achieved with 2-3 litres of Klean-Prep through the duodenal tube (according to standard protocols) to ensure complete bowel lavage (duration 3-4 hours).

Infusion

Finally, the filtered and mixed microbiota transplant (< 6 hours after processing) will be infused in the duodenum through a duodenal tube.

Escape medication

In case of nausea or vomiting anti-emetics can be used, intravenously or orally.

Functional Magnetic Resonance Imaging (fMRI)

Neuroimaging studies of visually presented food stimuli in patients with anorexia nervosa have demonstrated decreased activations in inferior parietal and visual occipital areas, and increased frontal activations relative to healthy persons[35]. Therefore, an fMRI will be performed in the fasted state at baseline and 6 and 12 weeks after intestinal microbiota transplantation . This technique is well implemented at the AMC department of Radiology (Dr M. Caan and Dr A. Nederveen) and is a useful method to assess CNS activation, defined as blood oxygen level dependent (BOLD) signal, in response to food pictures. The fMRI tasks consist of pictures selected from three different categories: (1) high-energy food items; (2) low-energy food items; (3) non-food items. The primary outcome is the difference in BOLD signal, i.e. in CNS activation, in predefined regions in the CNS in response to viewing food pictures[12].

Resting Energy Expenditure (REE)
REE will be measured at baseline and after 4 and 8 weeks. Oxygen consumption and CO_2 production will be measured during 20 minutes using a ventilated hood system. With these measurements the REE and respiratory quotient (RQ) can be calculated. The RQ represents the ratio of carbon dioxide exhaled to the amount of oxygen consumed by the individual and represents whole body substrate oxidation (glucose, fat and protein oxidation). The abbreviated Weir equation is used to calculate the 24-hour energy expenditure.

Physical activity energy expenditure (PAEE)
PAEE will be measured using a combined accelerometer and heart rate monitor (ActiHeart; CamNTech Ltd., Cambridge) which will be worn as a watch around the wrist by subjects for 7 days prior to beginning and end of study[36,37].

Bio Impedance Analysis (BIA)
Body composition will be measured using BIA. BIA determines the electrical impedance of an electric current through body tissues which can then be used to calculate an estimate of body fat.

Satiety test (Nutrient Drink Test)

We will perform a nutrient drink test 1 day before intestinal microbiota transplantation, 6 and 12 weeks after intestinal microbiota transplantation. First a venflon will be placed. Thereafter, subjects consume 120 mL nutrient drink (after a 12-hour overnight fast), containing 1.06 kcal/mL with 65% of carbohydrate, 20% of fat and 15% of protein, every 4 minutes until full. The nutrient drink is administered in a paper cup that is refilled every 4 minutes. At 5 minute intervals, subjects score fullness using a rating scale that combines verbal descriptors on a scale graded 0-5 (0:no symptoms, 1 first sensation of fullness, 2: mild, 3: moderate, 4: severe and 5: maximum or unbearable fullness). Subjects are told to stop when a score of 5 is obtained. Postprandial symptoms will be measured 30 minutes after completing the test with participants scoring each symptom of bloating, fullness, nausea and pain on a visual analogue scale with 100 mm lines and the words “unnoticeable” and “unbearable” as anchors. Furthermore, plasma CCK, PYY, ghrelin, serum glucose and immunoreactive insulin (IRI) responses will be measured at baseline as well as at 30, 60, 90 and 120 minutes therafter[38].

24h urine samples

24h urine samples will be collected by the patient at baseline and after 6 and 12 weeks to detect changes in protein excretion. Patients will be collecting their urine in the provided materials, (wearing gloves) and store it in the fridge (3-4ºC) until their visit.

Morning stool samples

Morning stool samples will be collected at baseline, 6 and 12 weeks to determine changes in gut microbiota composition. Samples will be taken by collection on toilet paper (by patient him/herself wearing gloves), divided over 3 Eppendorfs and directly frozen in fridge at home (-20ºC). Samples will be transported to AMC on icepacks. At the AMC, all samples will be stored at -80ºC. Changes in short chain fatty acid metabolism (acetate, propionate and butyrate) at baseline and after 6 and 12 weeks will be evaluated[39,40]. [41].Sample analysis will be done by HIT Chip flora mapping, an established sensitive RT-qPCR method which is developed for exact and sensitive enumeration of bacterial population [42,43].

Blood samples: plasma markers for satiety

Blood samples will be taken from all patients during at baseline and after 6 and 12 weeks during the mixed meal test to evaluate the effect of the intestinal microbiota transplantation on satiety (post-absorptive plasma markers for satiety: tryptophan, ghrelin, leptin, neuropeptide Y and orexin levels) and metabolic profile of glucose and insulin.

Questionnaires

At baseline, 6 and 12 weeks the following questionnaires:
- Visual analog scale (VAS), General food cravings questionnaire (G-FCQ),
Satiety Labeled Intensity Magnitude (SLIM)

The following questionnaires will be performed by a nurse from the psychiatry department:
- Psychological response: score on the Yale-Brown-Cornell Eating Disorder Scale (YBC-EDS), obsessive compulsive symptoms and/or compulsivity about feeding will be measured with the Yale Brown Obsessive Compulsive Scale (YBOCS) and assessment of eating behaviour with the Eating Disorder Inventory (EDI-II) and the Eating Disorder Examination Questionnaire (EDEQ).
- Quality of life (Eating Disorders Quality of Life, EDQOL)

6.4. Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. In this case, financial compensation will be calculated based on extent of participation. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

6.4.1. Specific criteria for withdrawal (if applicable)

Participants with any new condition during the study requiring hospital admission, change in drug prescriptions or likely to affect the outcome of this study will be withdrawn.
Participations will be withdrawn if instructions, as written in the protocol, won't be followed.

6.5. Replacement of individual subjects after withdrawal

Withdrawn subjects will be replaced by new subjects.

6.6. Follow-up of subjects withdrawn from treatment

If a subject decides to leave the study, the investigator will ask for the reason. Subjects withdrawn for medical reasons will be followed until the interfering condition has resolved or reached a stable state.

6.7. Premature termination of the study

In case of multiple SUSARS the study will be terminated.

7. EFFICACY MEASUREMENTS IMT

In order to evaluate the functional efficacy or positive therapeutic potential of allogenic IMT, patients with anorexia nervosa have to meet one of the following criteria:

1. **Physiological response**: Weight gain > 4% in 6 weeks.

   OR

2. **Psychological response**: Significant improvement* on eating disorder questionnaires assessing the psychological response and/or quality of life at 6 weeks.
   Psychological response is measured through scores of the Yale-Brown-Cornell Eating Disorder Scale (YBC-EDS), the Yale Brown Obsessive Compulsive Scale (YBOCS), and the Eating Disorder Inventory (EDI-II) and the Eating Disorder Examination Questionnaire (EDE-Q)).
   Quality of life is measured with the Eating Disorders Quality of Life (EDQOL) questionnaire.

***** AN patients will be asked to complete the questionnaires before and after (at 6 weeks and 12 weeks) receiving IMT~~FMT~~. For each AN patient, the difference in questionnaire score between these time-points (before receiving IMT~~FMT~~ vs. 6 and 12 weeks after receiving IMT) will be assessed. These will be interpreted separately in the light of each questionnaire. For example, in case of the YBC-EDS, a lower total YBC-EDS score or preoccupations and/or rituals subscale scores correlates with a lesser severity of the eating disorder[44,45]. The extent to which these scores change in patients that have received an

autologous transplant will be compared to those that have received allogeneic material. The appropriate statistical tests will then be performed to determine whether the differences in score-changes between these two groups are statistically significant ($p<0.05$).

A significant change in any of the questionnaires' used to assess psychological response or in the questionnaire assessing quality of life (total and/or sub-scores at six weeks post-IMT) will classify as a psychological response attributed to the IMT procedure. A "psychological response" can therefore be categorized as an improvement of eating disorder specific psychological functioning after (allogeneic) microbiota transplantation. Because we cannot be certain of the durability of IMT effects, we consider statistically significant changes at six weeks post-IMT to be a response, independent of the possible return to base-line values at 12 weeks post-IMT.

8. SAFETY REPORTING

8.1 Section 10 WMO event

In accordance to section 10, subsection 1, of the WMO, in the event of the trial proving to be significantly more unfavorable to the subject than the research protocol had suggested, the investigator is required to notify both the subject and the committee which was last to review the protocol in accordance with section 2 without delay, and apply to the said committee for a further review. Under such circumstances, performance of the trial will be suspended until such time as continuation is approved by the committee in question, unless suspension or cessation would be prejudicial to the health of the subject.

8.2 AEs, SAEs and SUSARs

8.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to [the investigational product / the experimental intervention]. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

8.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that at any dose:

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect;
  Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious adverse experience when, based upon appropriate medical judgement, the event may jeopardize the subject or may require an intervention to prevent one of the outcomes listed above.

Only SAEs that can be directly linked to the procedure, except for hospitalization due to fever, will be reported through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 15 days after the sponsor has first knowledge of the serious adverse reactions. Patients admitted to the hospital due to fever are recorded and will be reported to the METC periodically, every 3 months (line listing). All other SAEs will be recorded but not reported through the web portal *ToetsingOnline*.

SAEs that result in death or are life threatening should be reported expedited. The expedited reporting will occur not later than 7 days after the responsible investigator has first

knowledge of the adverse reaction. This is for a preliminary report with another 8 days for completion of the report.

8.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Adverse reactions are all untoward and unintended responses to the intestinal microbiota transplantation.

Unexpected adverse reactions are SUSARs if the following three conditions are met:

1. The event must be serious (see chapter 6.2.2);
2. There must be a certain degree of probability that the event is a harmful and an undesirable reaction to the intestinal microbiota transplantation.
3. The adverse reaction must be unexpected, that is to say, the nature and severity of the adverse reaction could not have been foreseen, using outcomes of previous studies exploring the effect of intestinal microbiota transplantation.

8.3 Annual safety report

Not applicable.

8.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.
SAEs need to be reported in ToetsingOnline till end of study within the Netherlands, as defined in the protocol.

9. STATISTICAL ANALYSIS

9.1 Primary study parameter(s)

All data will be analysed using SPSS for Windows, version 20.0 (SPSS Inc. Chicago, Illinois, USA). Multivariate analysis and ANOVA for repeated measures will be used. Wilcoxon's signed-rank test will be used to compare results between the study groups. Student T-test will be used to analyse the within group change. Data will be expressed as median and range. Spearman's rank test will be used to calculate correlations.

9.2 Secondary study parameter(s)

See paragraph 9.1

9.3 Other study parameters

No statistical tests will be performed on the baseline characteristics.

9.4 Interim analysis

No interim analysis will be performed.

10. ETHICAL CONSIDERATIONS

10.1 Regulation statement

This study will be performed according to the principles of the Declaration of Helsinki (October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO, March 2006).

10.2 Recruitment and consent

Participants will be recruited in three ways:

1) By treating physicians at the department of Psychiatry and Internal medicine of the AMC, Novarum (Amsterdam), the Ursula clinic (Leiden) and Rintveld institute (Zeist)

2) Via advertisements on online patient forums such as “ikookvanmij” and “ixtanoa”, after obtaining permission from the forum directory.

3) Donors will be recruited through advertisement in a local newspaper (Metro and de Telegraaf, editie Amsterdam).

All participations will be given oral and written information about the study by the investigator. Participants will be given at least three days to consider their participation before written informed consent is obtained.

10.3 Benefits and risks assessment, group relatedness

Risk assessment:

-The placing of the duodenal tube can be an unpleasant experience for the subjects, but there are no risks involved.

- No side effects of intestinal microbiota transplantation (FATLOSE 1 MEC, 07/114; FATLOSE 2 MEC 11/023; FEBALIGO MEC 2013_090) have been reported in our previous trials. Because a strict screening protocol is applied to donors of intestinal microbiota at the AMC, the risk of spreading potential pathogens during intestinal microbiota transplantation seems negligible and no long term effects have been reported at our clinic since 2007 (>300 intestinal microbiota transplantations performed). Since this is a new patient group (patients with anorexia nervosa) we classify this study as medium risk.

10.4 Compensation for injury

The investigator has a liability insurance that is in accordance with article 7, subsection 6 of the WMO.

1. €650.000,-- (i.e. six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
2. € 5.000.000,-- ,-- (i.e. five million Euro) for death or injury for all subjects who

participate in the Research;
3. € 7.500.000,-- (i.e. seven million five hundred thousand Euro) for the total damage incurred by the organization for all damage disclosed by scientific research for the Sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

10.5 Incentives (if applicable)

All participants will receive a financial compensation, patients 250 and donors 100 Euros, and a refund for travel costs.

11. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

11,1 Handling and storage of data and documents

The principle investigator will maintain a signature list of appropriately qualified persons to whom he delegated study duties. The investigator will maintain all CRF's, fMRI data files and all source documents that support the data collected from each subject and all study documents, and will treat these data with confidentially according to the Dutch Personal Data Protection Act. A subject identification code list will be drawn, and per subject data will be filed under the subject's unique code. The subject will be identified by this number for the duration of the trial. All the participating investigators have access to the source data. Blood samples for analysis and storage will be labelled with this code and visit number. Codes cannot be retraced to the corresponding subject without the identification code list. All essential documentation and human materials will be retained by the institution. Study documents will be archived for 15 years.

11.2 Amendments

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

-the safety or physical or mental integrity of the subjects of the trial;
-the scientific value of the trial;
-the conduct or management of the trial; or
-the quality or safety of any intervention used in the trial.

All amendments will be notified to the METC and to the competent authority.

11.3 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided to the DSMB on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

11.4 End of study report

The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.
In case the study is ended prematurely, the investigator will notify the accredited METC within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

11.5 Public disclosure and publication policy

The trial will be registered in the Netherland Trial Registry before study onset.

The results of this study will be submitted for publication in an international, peer-reviewed journal.

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