BMJ Open

# Protocol for a double-blinded randomised controlled trial to assess the effect of faecal microbiota transplantations on thyroid reserve in patients with subclinical autoimmune hypothyroidism in the Netherlands: the IMITHOT trial

Aline C Fenneman ,[1,2] Elena Rampanelli,[1] Anne H van der Spek,[2] Eric Fliers,[2] Max Nieuwdorp[1]

**To cite:** Fenneman AC, Rampanelli E, van der Spek AH, *et al*. Protocol for a double-blinded randomised controlled trial to assess the effect of faecal microbiota transplantations on thyroid reserve in patients with subclinical autoimmune hypothyroidism in the Netherlands: the IMITHOT trial. *BMJ Open* 2023;**13**:e073971. doi:10.1136/bmjopen-2023-073971

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (http://dx.doi.org/10.1136/bmjopen-2023-073971).

Received 23 March 2023
Accepted 22 August 2023



For numbered affiliations see end of article.

**Correspondence to**
Dr Aline C Fenneman;
a.fenneman@amsterdamumc.nl

## ABSTRACT

**Background** Hashimoto's thyroiditis (HT) is a common endocrine autoimmune disease affecting roughly 5% of the general population and involves life-long treatment with levothyroxine, as no curative treatment yet exists. Over the past decade, the crosstalk between gut microbiota and the host immune system has been well-recognised, identifying the gut microbiome as an important factor in host health and disease, including susceptibility to autoimmune diseases. Previous observational studies yielded a link between disruption of the gut microbiome composition and HT. This is the first study that investigates the potential of restoring a disrupted gut microbiome with faecal microbiota transplantations (FMTs) to halt disease progression and dampen autoimmunity.

**Methods and analysis** The IMITHOT trial is a randomised, double-blinded, placebo-controlled study evaluating either autologous or allogenic FMTs in medication-naïve patients with subclinical autoimmune hypothyroidism. In total, 34 patients will be enrolled to receive either three allogenic or autologous FMTs. FMT will be made of fresh stool and directly administered into the duodenum. Patients will be evaluated at baseline before the first FMT is administered and at 6, 12 and 24 months post-intervention to assess efficacy and adverse events. The primary outcome measure will be the net incremental increase (incremental area under the curve) on thyrotropin-stimulated free thyroxine and free triiodothyronine release at 6 and 12 months compared with baseline. Results will be disseminated via peer-reviewed journals and international conferences. The recruitment of the first patient and donor occurred on 18 December 2019.

**Ethics and dissemination** Ethics approval was obtained from the hospital Ethics Committee (Medical Ethics Committee) at Amsterdam University Medical Center. The trial's outcomes offer high-quality evidence that aids in unveiling distinct patterns within the gut microbiota potentially associated with improved thyroid function. Consequently, this may open avenues for the future clinical applications of microbial-targeted therapy in individuals at risk of developing overt HT.

**Trial registration number** NL7931.

## STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Medication-naïve patients with subclinical autoimmune hypothyroidism will undergo three faecal microbiota transplantations (FMTs) using either the same donor or participants' own stool to enhance the successful engraftment of the gut microbiota.
⇒ This study protocol lacks a control group without FMT intervention (eg, a group receiving a saline solution instead of faecal suspension).
⇒ Residual thyroid function will be measured using free thyroxine and free triiodothyronine excretion on recombinant TSH (Thyrogen) injection.
⇒ Participants will be followed for 2 years after the first FMT to study the sustainability of the faecal microbiota engraftment.
⇒ Patients reported outcomes will be measured with the validated thyroid-related quality of life (thyroid-specific patient-reported outcome) questionnaire.

## INTRODUCTION

Hashimoto's thyroiditis (HT), characterised by the progressive destruction of thyroid hormone-producing thyrocytes, is an autoimmune endocrine disorder resulting from genetic susceptibility accompanied by particular environmental factors. HT is the most common form of hypothyroidism in iodine-sufficient areas, affecting roughly 5% of the general population with a female predominance (8:1 female to male ratio).[1]

The natural course of HT is a slow development over the years, evolving from positive serum levels of antibodies to thyroid peroxidase (TPOAb) with serum thyroid hormone levels still within the reference range (euthyroidism) to subclinical (elevated thyroid stimulating hormone (TSH) with normal serum free thyroxine (fT4) levels) and eventually overt hypothyroidism (elevated TSH with decreased serum fT4 levels). Approximately 10% of the general population have positive TPOAb serum levels,[2 3] but not all will develop overt hypothyroidism. Previous studies have shown that the likelihood of developing overt hypothyroidism significantly rises in subjects with elevated TSH and positive TPOAb serum levels: a 20-year follow-up study revealed an OR of 28 (95% CI 22 to 65) and 173 (95% CI 81 to 370) in this specific group of women and men, respectively.[4] A different study showed that 66.6% of participants (aged 55 years or older) with a TSH level of ≥10 mIU/L progressed to overt hypothyroidism after an average of 18.3 months.[5] Moreover, another study demonstrated a significantly increased relative risk of 15.6 of developing overt hypothyroidism in patients with autoimmune subclinical hypothyroidism with a TSH level of ≥12 mIU/L, with an annual progression rate of 11.4±3.0%.[6] Thyroid ultrasound examination may provide additional information in patients with thyroid autoimmunity, as a hypoechoic and inhomogeneous ultrasound pattern with nodules is a risk factor for the development of HT.[7 8]

Current treatment of HT consists of life-long hormone substitution therapy with daily oral administration of the synthetic thyroid hormone levothyroxine (LT4). In 2021, over 500 000 people in the Netherlands were using LT4, making it one of the most prescribed medications in patients between 65 and 74 years (3.8%) in the Netherlands.[9] However, approximately 5–15% of patients with euthyroid HT receiving LT4 treatment still experience various persistent symptoms, with fatigue being the most significant.[10 11] The interpretation and optimal management of these symptoms are still being determined.[12] No curative treatment is available to restore normal thyroid function, as the underlying autoimmune aetiology is not fully understood.

The hallmark of HT is the drastic loss of thyroid hormone-producing follicular cells by T cell-mediated autoimmune responses. The loss of self-tolerance against the main autoantigen thyroid peroxidase (TPO) results in infiltration of the thyroid gland by mainly autoreactive T and B cells and is postulated to be driven by an overt activation of T helper (Th) type 1 (T1) and Th17 cells at the expense of the immunosuppressive activity of regulatory T cells.[13–16] This extensive stimulation is followed by predominantly lymphocytic infiltration of several B-cell phenotypes in the thyroid gland with well-defined germinal centres. In contrast, no distinct lymphocytic infiltration was found in the thyroid tissue of healthy controls.[13] Interestingly, while patients with untreated hypothyroid had a similar proportion of interleukin-10+regulatory B cells (Breg) as healthy individuals,[17] patients with euthyroid HT who received thyroxine treatment showed an increased proportion of functional Breg cells.[18] Therefore, our research aims to investigate the proportion of T and B cells, including Bregs, in both peripheral blood and thyroid gland tissue obtained through ultrasound-guided fine needle aspiration to gain a comprehensive understanding of the immune cell dynamics associated with HT.

The human gut microbiome (the collective genomic content of microorganisms) consists of $10^{13}$ to $10^{14}$ bacterial cells (microbiota). As the gut constitutes the largest immune component in humans (residing up to 70–80% of all immune cells), the gut microbiota, epithelial layer and mucosal immune system are closely connected.[19 20] The gut microbiome has now been identified as an important factor in regulating host health and disease, including the susceptibility to autoimmunity and the production of microbial-derived immunomodulatory metabolites.[21–24]

Previous studies,[25–31] including a recent systematic review,[32] have linked an altered intestinal microbiota composition (dysbiosis) to HT pathophysiology. However, these studies were primarily cross-sectional and observational in nature,[33] and causality has yet to be demonstrated. Two recent studies observed a significant positive (but minimal clinical) effect of synbiotic supplementation on serum thyroid markers in patients with hypothyroidism using LT4 compared with their placebo-treated counterparts. Unfortunately, both studies lacked information on gut microbiota composition and functionality before and after the intervention.[34 35] The present IMITHOT study is the first to test the effect of restoring intestinal homeostasis with healthy microbiota using faecal microbiota transplantations (FMTs) on thyroid function in medication-naïve patients with subclinical autoimmune hypothyroidism. Recently, our group has shown that this therapeutic approach is safe and effective in preserving endocrine function in autoimmune diabetes and halting disease progression.[36] Potential mechanisms underlying a beneficial effect of FMT include changes in the production of immunoregulatory metabolites by commensal gut microbes and the acquisition of an immunoregulatory phenotype of T cells trafficking through the intestinal lymphoid tissue.[37 38] Therefore, we hypothesise that changing the gut microbiota composition with multiple FMTs might dampen the autoimmunity of these T cells and may halt the destruction of the thyroid gland, thus delaying or even preventing the need for exogenous thyroid hormone supplementation with LT4 in patients at high risk of developing overt hypothyroidism. The study aim is to develop the first known treatment to prevent the development of autoimmune hypothyroidism by targeting microbial-immune interactions within the gut-thyroid axis.

## Primary objective

The study aims to investigate whether compositional changes of the gut microbiome induced by multiple FMTs from either allogenic (healthy) or autologous (own) donor stool, administrated via a nasoduodenal tube, have

beneficial effects on residual thyroid functions (fT4 and free triiodothyronine (fT3) excretion on recombinant TSH (rTSH) (Thyrogen) injection) in patients recently diagnosed with subclinical autoimmune hypothyroidism.

### Key secondary objectives

- Study the effect of FMTs on changes in immunophenotypes and cell activation in circulating peripheral blood mononuclear cells (PBMCs) and immune cells infiltrating the thyroid gland (retrieved by fine needle aspiration).
- Study the effect of FMT on oral and faecal microbiota composition as well as (microbiota-derived) fasted plasma metabolites measured at baseline and 6, 12 and 24 months follow-up.
- Study the effect of FMT on intestinal transit time, measured by the amount of ingested radiopaque markers (Transit-Pellets) seen on an abdominal X-ray.
- Study the effect of FMT on quality of life using the validated thyroid-specific patient-reported outcome (ThyPRO) questionnaire.
- Assess dietary intake via a food frequency questionnaire (via mijn.voedingscentrum.nl/nl/eetmeter).

## METHOD AND ANALYSES: PARTICIPANTS, INTERVENTIONS AND OUTCOMES

### Study design

The IMITHOT trial is a double-blinded, randomised-controlled, exploratory, single-centre trial. Each patient will receive three FMTs, with 2 months between each FMT. Patients will be randomised to one of the two following treatment arms (figures 1 and 2):

1. Three allogenic (healthy donor) faecal infusions at baseline, 8 and 16 weeks.
2. Three autologous (patients' own) faecal infusions at baseline, 8 and 16 weeks.

Eligible patients will be followed-up for 2 years after the first intervention to monitor residual thyroid function and ensure a high FMT engraftment success rate. This study protocol is reported per the Standard Protocol Items: Recommendations for Interventional Trials guidelines.[39]

This study will enable us to determine if gut microbiota plays an essential role in disease progression and, second, provides an indication of which microbiota components might predict a positive response to FMT in the pathophysiology of HT. A strength of this study protocol is that patients will receive multiple FMTs administered via a nasoduodenal tube to obtain a high bacterial strain engraftment success rate. Subsequently, the long follow-up period of 2 years allows us to assess the long-term impact and sustainability of FMT engraftment.

There is no established standard for determining the FMT treatment period. In our study, we have chosen to administer three FMTs every 2 months based on the treatment protocol employed in a recent clinical trial conducted by our group.[36] This trial investigated the efficacy of a similar study design in patients with new-onset

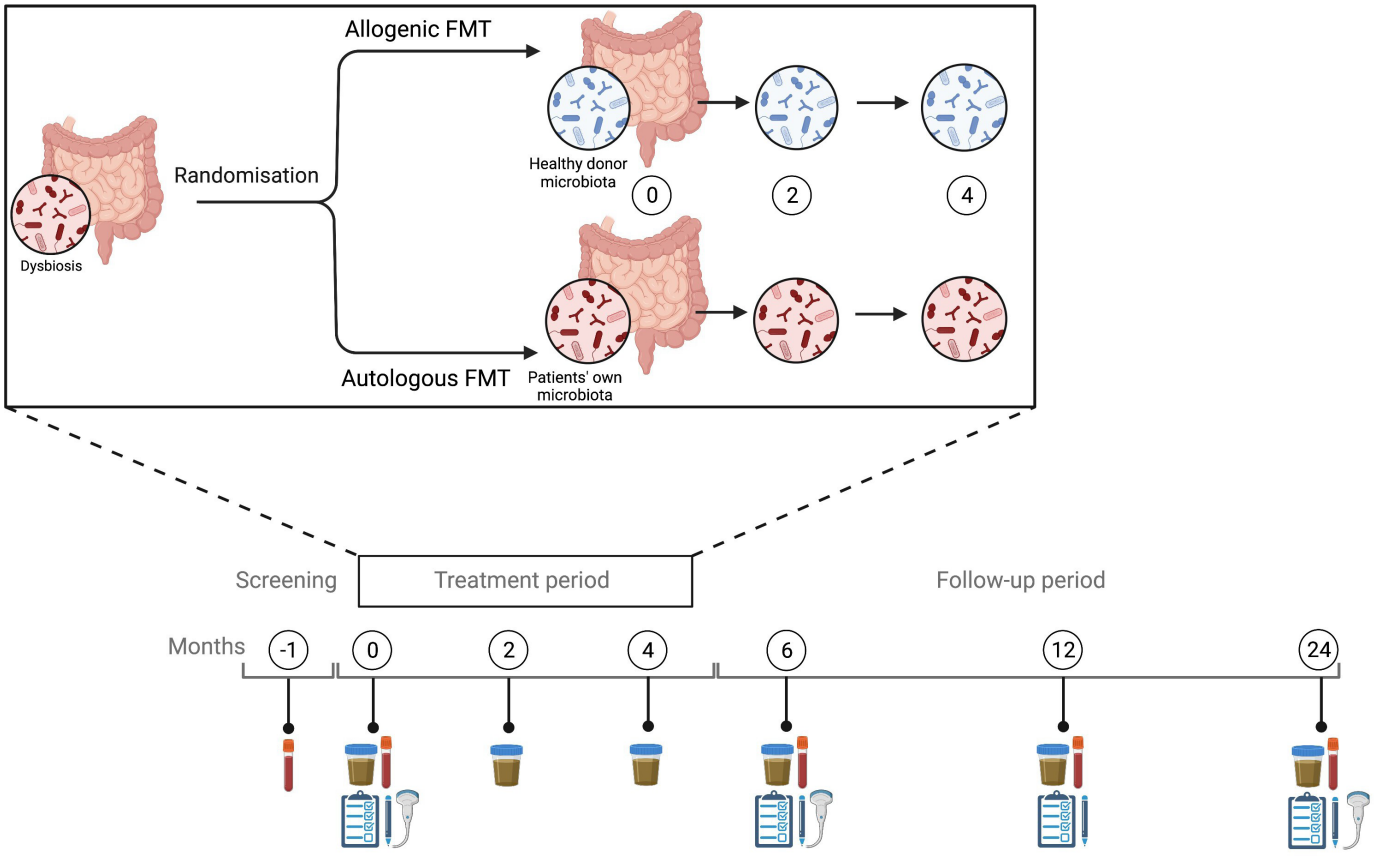


**Figure 1** Schematic overview of the study design. FMT, faecal microbiota transplantation.

| | Enrolment | Allocation | During treatment | | Follow-up | | |
|---|---|---|---|---|---|---|---|
| **TIMEPOINT** | ***T-4*** | **T0** | ***T2*** | ***T4*** | ***T6*** | ***T12*** | ***T24*** |
| | ***-4 weeks*** | ***Baseline*** | ***2 months*** | ***4 months*** | ***6 months*** | ***12 months*** | ***24 months*** |
| | **Screening patient** | **1st FMT** | **2nd FMT** | **3rd FMT** | | | |
| **ENROLMENT:** | | | | | | | |
| Eligibility screen | X | | | | | | |
| Informed consent | X | | | | | | |
| Allocation | | X | | | | | |
| **INTERVENTIONS:** | | | | | | | |
| *Allogenic FMT* | | ← | — | → | | | |
| *Autologous FMT* | | ← | — | → | | | |
| **ASSESSMENTS:** | | | | | | | |
| *rTSH test (residual thyroid function)* | | X | | | X | X | X |
| *Ultrasound-guided FNA thyroid gland* | | X | | | X | | X |
| *Oral and stool samples* | | X | X | X | X | X | X |
| *Fasted blood samples* | | X | X | X | X | X | X |
| *ThyPRO questionnaire* | | X | X | X | X | X | X |
| *Transit-Pellets radiopaque markers* | | X | | | X | X | X |

**Figure 2** Schematic overview of the study design according to the Standard Protocol Items: Recommendations for Interventional Trials guidelines. FMT, faecal microbiota transplantation; FNA, fine needle aspiration; rTSH, recombinant thyroid stimulating hormone; ThyPRO, thyroid-specific patient-reported outcome.

type 1 diabetes. Given the focus of our current study on another autoimmune endocrine disease targeting the thyroid gland, we have opted for the same treatment protocol.

Next, previous research by our group has shown that the beneficial effects of FMT are transient. We observed only a short-term improvement in insulin sensitivity, whereas no long-term effects were seen 18 weeks after FMT.[40] This finding suggests that performing FMT more frequently over an extended period may be beneficial. In line with this, a study by Li *et al*[41] indicated that the engraftment of donor bacterial strains could be transient, and individuals possess their own unique faecal core microbiome. Consequently, a single FMT may not lead to sustained changes in the intestinal microbiota composition. Another study demonstrated that three FMTs significantly improved long-term microbiota engraftment ($p<0.05$).[42] Thus, our decision to conduct three FMTs every 2 months in our study is based on the goal of achieving a higher engraftment rate and promoting more sustained changes in the intestinal microbiota. We acknowledge that further research is required to determine the optimal treatment period for FMT, and we will thoroughly evaluate the results of our study to contribute to the understanding of this question.

## Study setting

The participants are recruited through general practitioners affiliated with the Amsterdam University Medical Center (Amsterdam UMC), advertisements via posters, and the patient's association *Schildklier Organisatie Nederland*. All study interventions will be performed at a single centre, Amsterdam UMC, location AMC, the Netherlands. This is an academic centre with over 10 years of experience in the administration of FMTs.[36 43–45] The first patient and donor were recruited on 18 December 2019. The intended end date is December 2024.

## Eligibility criteria

### Inclusion criteria patients

The inclusion criteria for patients with subclinical autoimmune hypothyroid are as follows:

- Men and women between 18 and 70 years of age at the time of inclusion.
- Non-obese body mass index (BMI) (18–30 kg/m$^2$).
- Confirmed diagnosis of subclinical autoimmune hypothyroidism:
  - TSH≥10 mIU/L.
  - FT4 within normal reference values (12.0–22.0 pmol/L).
  - Anti-TPO positive (>60 kU/L).

  - History of at least three consecutive abnormal blood results, with the second test performed at least 3 months after the first test.
- Ability to give informed consent.
- Residing in the Netherlands.

Thus, only medication-naïve patients with relatively high TSH serum levels and positive TPOAb serum levels will be included in this study as these patients are less likely to normalise to a euthyroid state spontaneously,[8] and LT4 treatment might affect gut microbiota composition. If patients' serum fT4 level decreases during the study period or develop severe symptoms of hypothyroidism, we prioritise their well-being and refer them back to their own family doctor who can prescribe LT4 as appropriate. These patients are then excluded from our study.

The maximum age for inclusion is 70 years as TSH levels tend to increase with age. Consequently, the American Thyroid Association recommends raising the target serum TSH to 4–6 mIU/L in individuals aged 70–80.[46] Next, patients are randomised to either autologous or allogenic FMTs, but a control group without FMT intervention (eg, an observational control group) is lacking due to ethical reasons.

### Exclusion criteria patients

- Diagnosis or symptoms of other autoimmune diseases (eg, type 1 diabetes mellitus, coeliac disease, autoimmune gastritis, rheumatoid arthritis or inflammatory bowel diseases such as Crohn's disease and ulcerative colitis).
- Use of any medication, including proton pump inhibitors, antibiotics and probiotics/prebiotics in the past 3 months or during the study period.
- History of chronic diarrhoea (≥3 defecations/day for >4 weeks), chronic constipation (<2 defections/week for >3 months), or irritable bowel syndrome according to Rome IV criteria.
- Smoking or illicit drugs use (3,4-Methylenedioxyme thamphetamine (MDMA), amphetamine, cocaine, heroin, γ-hydroxybutyric acid (GHB)) in the past 3 months or during the study period.
- Use of >5 alcoholic units on an average daily basis in the past 3 months or during the study period.
- History of cholecystectomy.
- Prolonged compromised immunity (due to recent cytotoxic chemotherapy or HIV infection with a CD4<240/mm$^3$).

## Donors

Potential healthy stool donor candidates were recruited among the Amsterdam UMC non-healthcare workers and preclinical medical students. Informed consent was obtained after oral and written information about the screening and donation process. Financial compensation was offered for qualified donors (€50,- per donation). The screening process, inclusion and exclusion criteria are in accordance with the European consensus of FMT in clinical practice.[47] A comprehensive report on the specific donor screening process has been recently published[48] and can be found in the online supplemental material (online supplemental tables S1 and S2).

### Inclusion criteria donors

- Men and women of ≥18 years of age at the time of inclusion.
- Normal BMI (18–25 kg/m$^2$).
- Regular morning stool pattern.
- Ability to give informed consent.
- Residing in the Netherlands.

### Exclusion criteria donors

Exclusion criteria are presented in the online supplemental table S3.

## Interventions

A schematic overview of all study activities can be found in figures 1 and 2.

### Faecal microbiota transplantation

A fresh morning stool sample (100–200 g) will be collected by both the recipient and the healthy donor and processed directly (<2 hours) in the laboratory. An independent laboratory technician will randomise and blind the two collected samples. Either the autologous or allogenic faeces will be mixed with saline in a 1:1 ratio, homogenised and filtered. Meanwhile, a nasoduodenal tube is inserted with a CORTRAK* Enteral Access System, after which bowel lavage with 2–3 L of Klean-Prep (via the nasoduodenal tube) will be performed to ensure complete bowel lavage (3–4 hours). Finally, the FMT will be infused in the duodenum through the positioned tube (within 6 hours of the stool sample donation).

### Residual thyroid function test

The residual thyroid function will be measured via a dynamic thyroid function stimulation test. At baseline, 6, 12 and 24 months after the first FMT, a single intramuscular injection of 0.9 mg rTSH (Thyrogen) will be administrated, as previously described.[49] Blood samples will be drawn over the following 5 hours. The incremental area under the curve (iAUC) will be used to determine the net increase of the area under the concentration versus time between 0 and 300 min of serum fT4 and fT3 levels after subtracting the baseline value to account for the between-subject variation in fasting serum TSH levels. The iAUC values will be derived according to the trapezoidal rule.

### Ultrasound-guided fine needle aspiration thyroid gland

An ultrasound-guided fine needle aspiration (FNA) of the thyroid gland will be performed at baseline, 6, and 24 months by an experienced radiologist, who will also measure thyroid volume and echogenicity. Nodules (if present) will be scored using the American College of Radiology Thyroid Imaging Reporting & Data System (ACR TI-RADS) classification.[50] The FNA biopsy will be done from normal thyroid tissue.

### Gut microbiota composition

Morning oral and stool samples will be collected during each study visit to assess the effect of FMT on gut microbiota composition. An oral swab of the upper front teeth rim and saliva in overnight fasted patients who refrained from tooth brushing that morning are used to study the oral microbiota composition. All samples will be immediately stored at −80°C and analysed at the end of the study by sequencing the V3-V4 region of 16S rRNA genes with the Illumina MiSeq sequencer. Established protocols will be followed for DNA extraction to ensure the reliability and accuracy of the results, as described previously.[51 52]

### Intestinal transit time

Radiopaque Transit-Pellets markers will be used at baseline, 6, 12 and 24 months to measure colonic transit time, given that patients with (subclinical) hypothyroidism often experience constipation.[53 54] In short, the patient's intestinal transit time is measured by swallowing one capsule containing 10 markers each morning for 6 days before the study visit. An additional capsule is taken on the evening before the visit, 12 hours before the abdominal X-ray. This ensures a 144-hour interval between the first marker and the X-ray. The radiologist then counts the visible capsules on the X-ray image.

To calculate the colonic transit time, we determine the mean oro-anal transit time for the markers swallowed daily. With a daily dose of 10 markers, the transit time in days is obtained by dividing the number of markers counted from the X-ray film (M) by 10. This calculation is facilitated using the provided Medifactia tool.[54]

### Fasted blood drawn

Overnighted fasted blood is drawn at baseline, 6, 12 and 24 months to assess biochemistry, endocrinology, metabolomics and for PBMCs isolation. Samples will be centrifuged at 3000 RPM, at 4°C for 15 min and stored at −80°C.

## Outcomes

### Primary outcome

The primary effect parameter is the preserving (Thyrogen stimulated) fT4 and fT3 release at 6, 12 and 24 months compared with baseline (0 months). This dynamic thyroid functional endpoint is chosen because Thyrogen acts as an amplifier, which can magnify any underlying abnormality in thyroid hormone secretion by the thyroid gland, making it possible to detect subtle changes in thyroid functioning. Moreover, static thyroid serum markers (eg, a single, fasted measurement) could be affected by external factors, such as seasonal variation, the timing of blood draw, exercise, diet and lifestyle and BMI.[55–58] In a dynamic function test measuring the net incremental increase in the AUC, these factors may have less influence on the results.

### Secondary outcomes

- Gut microbiota composition by sequencing the V3-V4 region of 16S rRNA genes with the Illumina MiSeq sequencer (overall composition by alpha-diversity and beta-diversity indices, relative abundances of families, phyla and amplicon sequence variants, and principal component analysis of the taxonomic profiling).
- Profiling of immune cell subsets in PBMCs and thyroid tissue, using a single-cell high-dimensional profiling assay of Maxpar Direct Immune Profiling Assay.
- Fasting plasma targeted (microbial-derived) metabolomics will be measured by Metabolon (Durham, North Carolina, USA), using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (as previously described[36]). Raw data will be normalised to account for interday differences. The levels of each metabolite will be rescaled to set the median equal to 1 across all samples. Missing values, generally due to the sample measurement falling below the detection limit, will be imputed with the minimum observed value for the respective metabolites.
- Intestinal transit time will be measured by the amount of ingested radiopaque markers (Transit-Pellets) seen on an abdominal X-ray.
- The ThyPRO questionnaire will assess the patient-reported quality of life.
- Total caloric intake, macronutrients (carbohydrates, proteins, fats and fibres) and micronutrients (selenium and iodine, among others) will be reported by food frequency questionnaires (via mijn.voedingscentrum.nl/nl/eetmeter).

## Participant timeline

Potential patient participants will be screened at the first visit (V1) (figures 1 and 2, table 1). Baseline data will be collected on the first FMT (V2) day. The second (V3) and third (V4) FMT are scheduled for 2 months between each visit. Follow-up will be conducted for 2 years after the first FMT, during which the participants will visit thrice (V5–V7). The last visit (V7) will be completed 2 years after the first FMT. In accordance with the recruitment of the study protocol, the participants should not change their original eating habits and are not allowed to ingest prebiotic, probiotic or synbiotic supplements during the trial period. The follow-up time points of 6, 12 and 24 months were chosen for practical reasons, patient compliance and to minimise unnecessary burden on participants, based on previous studies from the same research group.[36]

### Patient allocation and blinding

Patients are allocated to either allogenic or autologous FMTs through computer-generated block randomisation (block size=4) in a 1:1 ratio to ensure equal sample sizes and avoid selection bias. After all patients have completed the study and the data have been locked, the investigator will unblind the materials.

## Patients and public involvement none

### Data collection and management

In the IMITHOT trial, data are collected during seven study visits, as defined in figures 1 and 2. Data collection

Table 1 Specification of patient screening

| Anthropometric measurements | |
|---|---|
| Demographics | Lifestyle (exercise, diet, alcohol intake) |
| Physical examination | |
| Serum screening | |
| Haematology | |
| Alanine aminotransferase | Gamma-glutamyl transferase |
| Alkaline phosphatase | Glucose (fasted) |
| Aspartate aminotransferase | Haemoglobin |
| Bilirubin | Creatinin |
| Complete bound count | Lipid spectrum: total cholesterol, HDL, LDL, Lp(a) |
| C reactive protein | Ureum |
| Estimated glomerular filtration rate | |
| Endocrinology | |
| Free triiodothyronine | Thyroid peroxidase antibodies |
| Free thyroxine | Thyroid stimulating hormone |
| Viruses (CLIA or PCR) | |
| Cytomegalovirus: IgG and IgM | Epstein-Barr Virus: VCA IgG and EBNA IgG |

CLIA, Chemiluminescence Immunoassay ; EBNA, Epstein-Barr virus nuclear antigen; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lp(a), Lipoprotein(a); VCA, viral-capsid antigen.

will be performed by trained local research staff and data entry in the Clinical EDC, CASTOR database. The Clinical Research Unit of the Amsterdam UMC will perform and monitor data entry and look after timely CRF (case report forms) delivery. Any other parameters necessary to evaluate the study endpoints and reason for end-of-protocol treatment are also documented. All subject data will be pseudonymised with a study code. The subjection identification log, which links subjects to the code, is kept in a trial file only accessible to study personnel. All research data will be stored for 15 years. An independent data monitoring committee will perform an interim analysis when the first 20 patients have finished the trial.

## Statistical methods

### Sample size calculation

As this is a phase III trial, a reliable sample size calculation is not feasible but is based on previous research.[36] A sample of 17 patients in each group (34 patients in total) is needed to provide 80% power to detect a 10% difference in the Thyrogen-stimulated fT4 and fT3 iAUC between treatment groups at 6, 12 and 24 months, with a two-sided test at $\alpha$=0.05 and assuming a 10% dropout. All power calculations were performed with an online power calculator (www.biomath.info/power/). This relatively small sample size allows us only to encounter major effects of the FMTs.

- Autologous arm: decline of Thyrogen-stimulated fT4 and fT4 $iAUC_{(0–300\ min)}$ from 150% to 100% at 12 and 24 months.
- Allogenic arm: decline of Thyrogen-stimulated fT4 and fT4 $iAUC_{(0–300\ min)}$ from 150% to 120% at 12 and 24 months.

### Statistical analysis

All statistical tests will be conducted as a two-sided test with a p value of <0.05 considered statistically significant. Unpaired Student's t-test or the Mann-Whitney U test will be used for baseline differences between the two groups, dependent on the distribution of the data. Data will be expressed as mean±the SD or the median with the IQR. The iAUC for the 5-hour residual thyroid function test (fT4 and fT3 after Thyrogen injection) will be calculated using the trapezoidal method. Depending on the data distribution, either the Pearson correlation or Spearman's rank test will be used for correlation analyses. A linear mixed model will be used to compare the primary endpoint, in which 'allocation' and 'time point' will be fixed effects and 'study ID' a random effect. The p value for the interaction between 'allocation' and 'time point' will be reported. Additionally, parameters will be compared between groups at various time points using the Mann-Whitney U test with multiplicity correction.

XGBoost machine learning classification algorithm will be applied to determine which microbial strains and/or metabolites predict the response to FMT treatment, immune profiling changes and residual thyroid function.

## ETHICS AND DISSEMINATION

Ethics approval was obtained in the Netherlands from the Medical Ethics Committee of the Amsterdam UMC, in accordance with the Declaration of Helsinki (updated version October 2013, Fortaleza Brasil) and with the Medical Research Involving Human Subjects Act (WMO). The trial is registered with the Netherlands Trial Register. A manuscript with the results of the primary study outcomes will be published in a peer-reviewed journal. All participants will provide written informed consent.

### Adverse events and safety

Patients will be submitted to multiple intramuscular injections of 0.9 mg Thyrogen, three FNAs of the thyroid gland (performed by an experienced radiologist), three insertions of a nasoduodenal tube and several vena punctions. Prior studies have shown that FMT is a safe therapy, and strict conditions apply for donor screening (online supplemental tables S1–S3). The possible complications are cited in the written patient information. Adverse events are defined as any undesirable experience occurring to a patient during the clinical trial, whether or not related to the trial and will be reported by the investigator.

A severe adverse event (SAE) is any medical occurrence or effect that at any dose results in:

- Death.
- Life-threatening situation (at the time of the event).
- Hospitalisation or prolongation of existing inpatients' hospitalisation.
- Persistent or significant disability.
- A congenital anomaly or birth defect.
- An SAE, but this was prevented due to timely intervention.

**Author affiliations**
[1]Department of (Experimental) Vascular Medicine, Amsterdam Cardiovascular Sciences (ACS), Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
[2]Department of Endocrinology and Metabolism, Amsterdam Gastroenterology Endocrinology & Metabolism (AGEM), AmsterdamUMC, University of Amsterdam, Amsterdam, The Netherlands

**Contributors** AF contributes to patient selection and will contribute to data analysis and manuscript writing. ER is contributing to laboratory procedures and will be contributing to data analysis and writing the manuscript. AHvdS will be contributing to data analysis and writing the manuscript. MN and EF have written the protocol.

**Funding** The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

**Competing interests** MN is co-founder and member of the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands. None of these are directly relevant to the current paper. There are no patents, products in development or marketed products to declare. The other authors declare no competing financial interests.

**Patient and public involvement** Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

**Patient consent for publication** Not applicable.

**Provenance and peer review** Not commissioned; externally peer reviewed.



**ORCID iD**
Aline C Fenneman http://orcid.org/0000-0003-4872-9493

## REFERENCES

1 Taylor PN, Albrecht D, Scholz A, *et al*. Global epidemiology of hyperthyroidism and hypothyroidism. *Nat Rev Endocrinol* 2018;14:301–16.
2 Fenneman AC, Boulund U, Collard D, *et al*. n.d. Compositional disruption of the gut microbiota in a multi-ethnic euthyroid population with thyroid autoimmunity: the HELIUS study. *SSRN Journal* 10.2139/ssrn.4309030 Available: https://ssrn.com/abstract=4309030
3 Amouzegar A, Gharibzadeh S, Kazemian E, *et al*. The prevalence, incidence and natural course of positive Antithyroperoxidase antibodies in a population-based study: Tehran thyroid study. *PLoS One* 2017;12:e0169283.
4 Vanderpump MPJ, Tunbrldge WMG, French JM, *et al*. The incidence of thyroid disorders in the community: A twenty-year follow-up of the Whickham survey. *Clin Endocrinol* 1995;43:55–68.
5 Díez JJ, Iglesias P. Spontaneous Subclinical hypothyroidism in patients older than 55 years: an analysis of natural course and risk factors for the development of overt thyroid failure. *J Clin Endocrinol Metab* 2004;89:4890–7.
6 Huber G, Staub J-J, Meier C, *et al*. Prospective study of the spontaneous course of Subclinical hypothyroidism: Prognostic value of Thyrotropin, thyroid reserve, and thyroid antibodies. *J Clin Endocrinol Metab* 2002;87:3221–6.
7 Ceylan I, Yener S, Bayraktar F, *et al*. Roles of ultrasound and power Doppler ultrasound for diagnosis of Hashimoto thyroiditis in anti-thyroid marker-positive euthyroid subjects. *Quant Imaging Med Surg* 2014;4:232–8.
8 Peeters RP. Subclinical hypothyroidism. *N Engl J Med* 2017;377:1404.
9 Nielen M, Hek K, Schermer J. n.d. Jaarcijfers Aandoeningen: Incidenties en Prevalenties. Uit: Nivel Zorg. 2020. Available: www.nivel.nl/nl/nivel-zorgregistraties-eerste-lijn/jaarcijfers-aandoeningen-incidenties-en-prevalenties
10 Wiersinga WM, Duntas L, Fadeyev V, *et al*. ETA guidelines: the use of L-T4 + L-T3 in the treatment of hypothyroidism. *Eur Thyroid J* 2012;1:55–71.
11 Perros P, Van Der Feltz-Cornelis C, Papini E, *et al*. The enigma of persistent symptoms in hypothyroid patients treated with Levothyroxine: A narrative review. *Clin Endocrinol (Oxf)* 2023;98:461–8.
12 Jansen HI, Boelen A, Heijboer AC, *et al*. Hypothyroidism: the difficulty in attributing symptoms to their underlying cause. *Front Endocrinol (Lausanne)* 2023;14:1130661.
13 Zha B, Huang X, Lin J, *et al*. Distribution of lymphocyte subpopulations in thyroid glands of human autoimmune thyroid disease. *J Clin Lab Anal* 2014;28:249–54.
14 Pyzik A, Grywalska E, Matyjaszek-Matuszek B, *et al*. Immune disorders in Hashimoto's thyroiditis: what do we know so far *J Immunol Res* 2015;2015:979167.
15 Marazuela M, García-López MA, Figueroa-Vega N, *et al*. Regulatory T cells in human autoimmune thyroid disease. *J Clin Endocrinol Metab* 2006;91:3639–46.
16 Rydzewska M, Jaromin M, Pasierowska IE, *et al*. Role of the T and B lymphocytes in pathogenesis of autoimmune thyroid diseases. *Thyroid Res* 2018;11:2.
17 Kristensen B, Hegedüs L, Lundy SK, *et al*. Characterization of regulatory B cells in graves' disease and Hashimoto's thyroiditis. *PLoS One* 2015;10:e0127949.
18 Santaguida MG, Gatto I, Mangino G, *et al*. BREG cells in Hashimoto's thyroiditis isolated or associated to further organ-specific autoimmune diseases. *Clin Immunol* 2017;184:42–7.
19 Hooper LV, Littman DR, Macpherson AJ. Interactions between the Microbiota and the immune system. *Science* 2012;336:1268–73.
20 Lee YK, Mazmanian SK. Has the Microbiota played a critical role in the evolution of the adaptive immune system *Science* 2010;330:1768–73.
21 Sekirov I, Russell SL, Antunes LCM, *et al*. Gut Microbiota in health and disease. *Physiological Reviews* 2010;90:859–904.
22 Cho I, Blaser MJ. The human Microbiome: at the interface of health and disease. *Nat Rev Genet* 2012;13:260–70.
23 Round JL, Mazmanian SK. The gut Microbiome shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009;9:313–23.
24 Levy M, Kolodziejczyk AA, Thaiss CA, *et al*. Dysbiosis and the immune system. *Nat Rev Immunol* 2017;17:219–32.
25 Ishaq HM, Mohammad IS, Guo H, *et al*. Molecular estimation of alteration in intestinal microbial composition in Hashimoto's thyroiditis patients. *Biomed Pharmacother* 2017;95:865–74.
26 Zhao F, Feng J, Li J, *et al*. Alterations of the gut Microbiota in Hashimoto's thyroiditis patients. *Thyroid* 2018;28:175–86.
27 Cornejo-Pareja I, Ruiz-Limón P, Gómez-Pérez AM, *et al*. Differential microbial pattern description in subjects with autoimmune-based thyroid diseases: A pilot study. *J Pers Med* 2020;10:192.
28 Su X, Zhao Y, Li Y, *et al*. Gut Dysbiosis is associated with primary hypothyroidism with interaction on gut-thyroid axis. *Clin Sci* 2020;134:1521–35.
29 Liu S, An Y, Cao B, *et al*. The composition of gut Microbiota in patients bearing Hashimoto's thyroiditis with Euthyroidism and hypothyroidism. *Int J Endocrinol* 2020;2020:5036959.

30 Cayres L de F, de Salis LVV, Rodrigues GSP, *et al*. Detection of alterations in the gut Microbiota and intestinal permeability in patients with Hashimoto thyroiditis. *Front Immunol* 2021;12:579140.
31 El-Zawawy HT, Ahmed SM, El-Attar EA, *et al*. Study of gut Microbiome in Egyptian patients with autoimmune thyroid diseases. *Int J Clin Pract* 2021;75:e14038.
32 Gong B, Wang C, Meng F, *et al*. Association between gut Microbiota and autoimmune thyroid disease: A systematic review and meta-analysis. *Front Endocrinol (Lausanne)* 2021;12:774362.
33 Fenneman AC, Bruinstroop E, Nieuwdorp M, *et al*. A comprehensive review of thyroid hormone metabolism in the gut and its clinical implications. *Thyroid* 2023;33:32–44.
34 Talebi S, Karimifar M, Heidari Z, *et al*. The effects of Synbiotic supplementation on thyroid function and inflammation in hypothyroid patients: A randomized, double-blind, placebo-controlled trial. *Complementary Therapies in Medicine* 2020;48:102234.
35 Ramezani M, Reisian M, Sajadi Hezaveh Z, *et al*. The effect of Synbiotic supplementation on hypothyroidism: A randomized double-blind placebo controlled clinical trial. *PLoS ONE* 2023;18:e0277213.
36 de Groot P, Nikolic T, Pellegrini S, *et al*. Faecal Microbiota transplantation HALTS progression of human new-onset type 1 diabetes in a randomised controlled trial. *Gut* 2021;70:92–105.
37 Esplugues E, Huber S, Gagliani N, *et al*. Control of Th17 cells occurs in the small intestine. *Nature* 2011;475:514–8.
38 Okada M, Zhang V, Loaiza Naranjo JD, *et al*. Islet-specific Cd8+ T cells gain Effector function in the gut Lymphoid tissues via bystander activation not molecular Mimicry. *Immunol Cell Biol* 2023;101:36–48.
39 Chan A-W, Tetzlaff JM, Gøtzsche PC, *et al*. SPIRIT 2013 explanation and elaboration: guidance for protocols of clinical trials. *BMJ* 2013;346:e7586.
40 Kootte RS, Levin E, Salojärvi J, *et al*. Improvement of insulin sensitivity after lean donor Feces in metabolic syndrome is driven by baseline intestinal Microbiota composition. *Cell Metab* 2017;26:611–9.
41 Li SS, Zhu A, Benes V, *et al*. Durable coexistence of donor and recipient strains after fecal Microbiota transplantation. *Science* 2016;352:586–9.
42 Ng SC, Xu Z, Mak JWY, *et al*. Microbiota Engraftment after Faecal Microbiota transplantation in obese subjects with type 2 diabetes: A 24-week, double-blind, randomised controlled trial. *Gut* 2022;71:716–23.
43 Witjes JJ, Smits LP, Pekmez CT, *et al*. Donor fecal Microbiota transplantation alters gut Microbiota and metabolites in obese individuals with Steatohepatitis. *Hepatol Commun* 2020;4:1578–90.
44 Koopen AM, Almeida EL, Attaye I, *et al*. Effect of fecal Microbiota transplantation combined with Mediterranean diet on insulin sensitivity in subjects with metabolic syndrome. *Front Microbiol* 2021;12:662159.
45 Vrieze A, Van Nood E, Holleman F, *et al*. Transfer of intestinal Microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* 2012;143:913–6.
46 Garber JR, Cobin RH, Gharib H, *et al*. Clinical practice guidelines for hypothyroidism in adults: cosponsored by the American Association of clinical Endocrinologists and the American thyroid Association. *Thyroid* 2012;22:1200–35.
47 Cammarota G, Ianiro G, Tilg H, *et al*. European consensus conference on Faecal Microbiota transplantation in clinical practice. *Gut* 2017;66:569–80.
48 Bénard MV, de Bruijn CMA, Fenneman AC, *et al*. Challenges and costs of donor screening for fecal Microbiota Transplantations. *PLoS One* 2022;17:e0276323.
49 Nielsen VE, Bonnema SJ, Hegedüs L. Effects of 0.9 mg recombinant human Thyrotropin on thyroid size and function in normal subjects: a randomized, double-blind, cross-over trial. *J Clin Endocrinol Metab* 2004;89:2242–7.
50 Tessler FN, Middleton WD, Grant EG, *et al*. ACR thyroid imaging, reporting and data system (TI-RADS): white paper of the ACR TI-RADS committee. *J Am Coll Radiol* 2017;14:587–95.
51 Deschasaux M, Bouter KE, Prodan A, *et al*. Depicting the composition of gut Microbiota in a population with varied ethnic origins but shared geography. *Nat Med* 2018;24:1526–31.
52 Mobini R, Tremaroli V, Ståhlman M, *et al*. Metabolic effects of Lactobacillus Reuteri DSM 17938 in people with type 2 diabetes: A randomized controlled trial. *Diabetes Obes Metab* 2017;19:579–89.
53 Abrahamsson H, Antov S, Bosaeus I. Gastrointestinal and Colonic segmental transit time evaluated by a single abdominal X-ray in healthy subjects and Constipated patients. *Scandinavian Journal of Gastroenterology* 1988;23:72–80.
54 Abrahamsson H. Measurement of Colonic transit time with the transit-Pelletstm method. n.d. Available: https://aprmedtech.com/wp-content/uploads/2015/10/Information-material-Complete-version.pdf
55 Kuzmenko NV, Tsyrlin VA, Pliss MG, *et al*. Seasonal variations in levels of human thyroid-stimulating hormone and thyroid hormones: a meta-analysis. *Chronobiol Int* 2021;38:301–17.
56 Helmreich DL, Tylee D. Thyroid hormone regulation by stress and behavioral differences in adult male rats. *Horm Behav* 2011;60:284–91.
57 Fu J, Zhang L, An Y, *et al*. Association between body mass index and thyroid function in euthyroid Chinese adults. *Med Sci Monit* 2021;27:e930865.
58 Babić Leko M, Gunjača I, Pleić N, *et al*. Environmental factors affecting thyroid-stimulating hormone and thyroid hormone levels. *IJMS* 2021;22:6521.

*Table S1. Specification of donor screening*

| | |
|---|---|
| **Anthropometric measurements** | |
| Demographics | Lifestyle (exercise, diet, alcohol intake) |
| Physical examination | |
| **Feces screening** | |
| Calprotectin (ELISA) | |
| **Bacteria** (PCR or stool antigen detection) | |
| Clostridium difficile (GDH and toxine) | Salmonella spp. |
| Helicobacter pylori | Shiga toxin-producing Escherichia coli 1 and 2 (STEC) |
| Pathogenic Campylobacter spp. | Shigella spp (EIEC) |
| Pleisiomonas shigelloides | Yersinia enterocolitica |
| **Multidrug-resistant organisms** (culture) | |
| Carbapenem-resistant Enterobacteriaceae (CRE) | Methicillin-resistant Staphylococcus aureus (MSRA) |
| ESBL-producing Enterbactereacceae | Vancomycin-resistant Enterococcus |
| Multi-drugs resistant Gram-negatives (MRGN) 3 | MGNR 4 |
| **Viruses** (RNA or DNA PCR) | |
| Adenovirus non-40/41 | Norovirus Type I and II |
| Adenovirus type 40/41 | Parechovirus |
| Astrovirus | Rotavirus |
| Enterovirus | Sapovirus |
| Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) | Hepatitis E virus |
| **Parasites** (PCR and/or microscopic evaluation) | |
| Blastocystis spp. | Entamoebe moshkovski |
| Cryptosporidium spp. | Entamoeba pelecki |
| Cyclospora | Giardia lamblia |
| Dientamoeba fragilis | Iodamoeba bütschlii |
| Endolimax nana | Isospora spp. |
| Entamoeba coli | Larvae |
| Entamoebe dispar | Microsporidium spp. |
| Entamoebe gingivalis | Parasitic worm eggs (Ridley) |
| Entamoebe hartmann | Protozoan Cysts and Oocysts (Ridley) |
| Entamoebe histolytica 1 | |
| **Serum screening** | |
| **Hematology** | |
| Alanine aminotransferase (ALAT) | Complete Bound Count (CBC) |
| Alkaline phosphatase (AF) | C-reactive protein (CRP) |
| Aspartate aminotransferase (ASAT) | Estimated Glomerular Filtration Rate (eGFR) |
| Bilirubin | Kreatinin |
| Gamma-glutamyl transferase (GGT) | Lipid spectrum |
| Hemoglobin A1c (HbA1c) | Ureum |
| **Endocrinology** | |
| Free thyroxine (FT4) | |
| Thyroid peroxidase antibodies (TPOAb) | |
| Thyroid stimulating hormone (TSH) | |
| **Bacteria** (ELISA) | |
| Treponema pallidum | |

| | |
|---|---|
| **Viruses** (CLIA or PCR) | |
| Cytomegalovirus (CMV): IgG and IgM | Hepatitis C virus: HCV IgG total |
| Epstein-Barr Virus (EBV): VCA IgG and EBNA IgG | Human immunodeficiency virus (HIV): Ag and Ab |
| Hepatitis A virus: Ig total and IgM | Human T-lymphocytic virus Type I and II (HTLV) |
| Hepatitis B virus: HBsAg, HBcore IgG total, and anti HbsAg | |
| **Parasites** (antibodies) | |
| Strongyloides stercoralis | |

*Table S2. Time interval of donor rescreening*

| Screening | Every 6 months | Prior every individual FMT | 60-days |
|---|---|---|---|
| Short rescreening questionnaire | | X | |
| Extensive screening questionnaire | X | | |
| Feces | | | |
| Parasites | X | | |
| Bacteria | X | | |
| Viruses | X | | |
| Calprotectine | X | | |
| SARS-CoV-2 | X | | X |
| Multidrugs resistant bacteria | X | | X |
| Serum | | | |
| Haematology | X | | |
| Endocrinology | X | | |
| Bacteria (ELISA) | X | | |
| Viral loads (CLIA or PCR) | X | | |
| Parasites (ELISA) | X | | |

7 *Table S3. Inclusion and exclusion criteria for healthy donors*

| **Healthy donors** |
|---|
| *Inclusion criteria* |
| Males and females of ≥ 18 years of age at the time of inclusion; |
| Normal BMI (18 – 25 kg/m$^2$); |
| Regular morning stool pattern; |
| Ability to give informed consent. |
| Residing in the Netherlands |
| *Exclusion criteria* |
| Use of any medication, including proton pump inhibitors, antibiotics, and pro-/prebiotics in the past three months or during the study period; |
| Following specific diets, including vegan, keto, and paleo diets; |
| History of, or known exposure to HIV, hepatitis B (HBV) or C (HCV) virus, syphilis, human T-lymphotropic virus (HTLV) I and II, malaria, trypanosomiasis, tuberculosis; |
| Known systemic infection not controlled at the time of donation; |
| Smoking or illicit drugs use (MDMA, amphetamine, cocaine, heroin, GHB) in the past three months or during the study visit; |
| Use of >5 alcoholic units on an average daily basis in the past three months or during the study period; |
| History of cholecystectomy; |
| Risky sexual behavior, including anonymous sexual contacts; contacts with prostitutes, drug addicts, individuals with HIV, viral hepatitis, or syphilis; work as a prostitute; history of a sexually transmittable disease; |
| Previous reception of tissue/organ transplant; |
| Previous (<12 months) reception of blood products; |
| Recent (<6 moths) body tattoo, piercing, earring, or acupuncture; |
| Recent (<6 months) needles tick accident; |
| Recent (<6 months) medical treatment in poor hygienic conditions; |
| Risk of transmission of diseases caused by prions; |
| Recent parasitosis or infection from rotavirus, Giardia lamblia, and other microbes with gastrointestinal (GI) involvement; |
| Recent travel in tropical countries, countries at high risk of communicable diseases, or traveler's diarrhea (period based on recommendations Sanquin for blood donors) |
| Recent (<6 months) history of vaccination with a live attenuated virus, if there is a possible risk of transmission; |
| Healthcare providers having frequent patient contact (to exclude the risk of transmission of multidrug-resistant organisms); |
| Individuals working with animals (to exclude the risk of transmission of zoonotic infections); |
| History of IBS (according to Rome IV criteria), IBD, functional chronic constipation, coeliac disease, and other chronic GI disorders; |
| History of chronic, systemic autoimmune disorders with GI involvement; |
| History of, or high risk for, GI cancer or polyposis; |
| Recent (< 6 months) appearance of diarrhea (≥3 stools/day) or hematochezia; |

| |
|---|
| History of neurological/neurodegenerative disorders; |
| History of psychiatric conditions; |
| Presence of chronic low-grade inflammation or metabolic syndrome (NCEP criteria) |
| Presence of T1D, T2D, or hypertension; |
| History of cholecystectomy; |
| Positive serologic test for HIV 1/2, hepatitis A virus (HAV), HBV, HCV, hepatitis E virus (HEV), active cytomegalovirus (CMV) or active Epstein–Barr virus (EBV), Strongyloides or lues; |
| Presence of faecal bacterial pathogens Salmonella spp., Shigella spp., Campylobacter spp., Yersinia spp., C. difficile, H. pylori, shigatoxigenic Escherichia coli (STEC), Aeromonas spp. or Pleisiomonas Shigelloides in faeces; |
| Positive Dual Faeces Test (DFT) for Giardia Lamblia, Dientamoebe fragilis, Entamoeba histolytica, Microsporidium spp., Cryptosporidium spp., Cyclospora, Isospora or Blastocystis Hominis. Positive microscopic exam for eggs, cysts, and larvae (*e.g.*, helminth eggs) |
| Presence of extended-spectrum beta-lactamase (ESBL) producers, Carbapenem-resistant Enterobacteriaceae (CRE), vancomycin-resistant enterococci (VRE), or methicillin-resistant Staphylococcus aureus (MRSA) in faeces; |
| Presence of Rotavirus, Norovirus I/II, Enterovirus, Parechovirus, Astrovirus, Sapovirus, or Adenovirus in faeces; |
| Presence of SARS-Cov2 in faeces; |
| Abnormal liver or renal function (creatinine >110 µmol/l, ureum >8,2 mmol/l, ASAT >40 U/L, ALAT >45 U/L, AF >120 U/L, GGT >60 U/L, bilirubin >17µmol/L) or impaired immunity (CRP >5 mg/L, haemoglobin <8,5 mmol/L, MCV 80-100 fL, leukocytes 4,0-10,5 $x10^9$/L, thrombocytes 150-400 $x10^9$/L). |
| Abnormal thyroid function (TSH < 0.5 or > 5.0 mU/L; FT4 < 12.0 or > 22.0 pmol/L; anti-TPO antibodies >60 kU/L). |

8

9