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Protocol for a multicentre cross-sectional, longitudinal study in rheumatoid arthritis and Parkinson's disease patients analysing the relation between the gut microbiome, fasting and immune status (ExpoBiome)

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4 1 **Protocol for a multicentre cross-sectional, longitudinal study in**
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6 2 **rheumatoid arthritis and Parkinson's disease patients analysing the**
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8 3 **relation between the gut microbiome, fasting and immune status**
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10 **(ExpoBiome)**
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Abstract

Introduction

Chronic inflammatory diseases such as rheumatoid arthritis (RA) and neurodegenerative disorders like Parkinson's disease (PD) have recently been associated with a decreased diversity in the gut microbiome, emerging as key driver of various diseases. The specific interactions between gut-borne microorganisms and host pathophysiology remain largely unclear so far. The microbiome can be modulated by interventions comprising modulated nutrition and food intake.

The aim of our clinical study is to examine (1) the effects of prolonged fasting and time-restricted eating (TRE) on the outcome parameters and the immunophenotypes of RA and PD with (2) special consideration of microbial taxa and molecules associated with the changes expected in (1) and (3) to identify factors that impact the disease course and treatment by in depth screening of microorganisms and molecules in personalised HuMiX gut-on-chip models, ideally to find novel targets for anti-inflammatory therapy.

Methods and Analysis

This trial is an open-label, multicentre, controlled clinical trial consisting of a cross-sectional and a longitudinal study. A total of 180 patients is recruited. For the cross-sectional study, patients with PD, patients with RA and healthy controls are recruited at two different, specialized clinical sites. For the longitudinal part, patients with PD and RA undergo 5-7 days of prolonged fasting (PF) followed by TRE (16:8) for a period of 12 months. One baseline visit takes place before the PF intervention and 10 follow-up visits will follow over a period of 12 months.

Ethics and dissemination

Ethical approval was obtained to plan and conduct the trial from the institutional review board of the Charité-Universitätsmedizin Berlin (EA1/204/19), the ethics committee of the state medical association (Landesärztekammer) of Hessen (2021-2230-zvBO) and the Ethics Review Panel (ERP) of the University of Luxembourg (ERP 21-001-A ExpoBiome). The results of this study will be disseminated through peer-reviewed publications and scientific presentations.

Trial registration number at clinicaltrials.gov:

NCT04847011

Key words: Microbiome, fasting therapy, intermittent fasting, time restricted eating, chronic disease, rheumatoid arthritis, Parkinson's disease, nutrition, chronic diseases, ExpoBiome, inflammation, gut on a chip, HuMiX, immunophenotype

91 Strengths and limitations of the study

- 92 • The participants of the longitudinal study will be closely monitored for 12 months and
93 routine blood parameters as well as anthropometric data and questionnaires will be
94 precisely documented
- 95 • This study will identify novel microbiome-derived common and disease-associated
96 molecules involved in immune system modulation in two major chronic diseases: RA
97 and PD.
- 98 • This study aims at also identifying novel targeted pathways to control chronic
99 inflammatory conditions in the future
- 100 • A limitation is the heterogeneity of the cohorts regarding age and sex, which is due to
101 the prevalence of the diseases: RA is more common in women, while PD is more
102 common in men and has a later disease onset.
- 103 • A bias exists in choosing RA and PD as chronic disorders to study immunophenotypes
104 although generalisable results are targeted

106 Introduction (1018)

107
108 The human microbiome is emerging as a key driver of various diseases through its complex of
109 distinct yet connected biomolecules (referred to as the “*expobiome*”)[1, 2]. The expobiome is
110 comprised of a diverse set of nucleic acids, polypeptides and metabolites which, in the gut
111 alone, are present in substantial concentrations[1]. However, the specific interactions
112 between gut-borne microorganisms and host (patho)physiology remain largely unknown.
113 Although host genetics shape the composition of the gut microbiome, the latter is particularly
114 influenced by non-genetic factors such as lifestyle and diet[3, 4]. Therefore, the microbiome
115 is a plausible target to modify health outcomes.

116
117 Individuals suffering from chronic diseases, including autoimmune, metabolic, and
118 neurodegenerative diseases as well as cancer, often present alterations from a gut
119 microbiome composition associated with health. These shifts are typically characterised by
120 an overgrowth of one or several microbial species with likely adverse effects including
121 pathobionts, as well as a decrease in beneficial taxa[5]. Such imbalances are referred to as
122 dysbiosis. Although structural microbiome changes are clearly detectable, the mechanistic or
123 functional consequences of dysbiosis are still largely unknown. They may, however, result in
124 dysregulated interactions with the immune system[6]. Considering the intricacy of the
125 immune system, the question arises whether the observed microbiome changes are cause or
126 consequence of disease. This implies that, in addition to the genetic predisposition of the
127 host, the gut microbiome needs to be considered as a potential pathogenic factor or major
128 driver in disease onset and course[3, 4].

129
130 RA and PD are two specific examples representing dysregulated microbiome-immune system
131 interactions [7, 8]. RA is a multifactorial, chronic and systemic autoimmune disease, primarily
132 affecting the lining of the synovial joints with a higher risk and younger age for disease onset
133 in women and a global prevalence of 1%[9, 10]. The exact disease pathogenesis is still unclear
134 and no cure for RA currently exists. In addition to the common local articular symptoms of

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3 135 RA, systemic comorbidities can affect the vasculature, metabolism and bones[11]. Besides
4 136 various environmental risk factors e.g. smoking and a Western diet, the host microbiome is
5 137 associated with the pathophysiology of the disease[12]. The diversity of the gut microbiome
6 138 has been reported to be decreased in individuals with RA, compared with the general
7 139 population, and is correlated with disease duration and autoantibody levels[13]. Studies in
8 140 murine models also report that autoimmune arthritis is strongly attenuated under germ-free
9 141 conditions[14]. The introduction of specific bacteria, e.g. segmented filamentous bacteria,
10 142 into germ-free animals or oral infection with *Porphyromonas gingivalis* drive autoimmune
11 143 arthritis through activation of T helper cells[14]. Several different taxa, including *Prevotella*
12 144 *copri*, *Lactobacillus* spp. and *Colinsella* spp. are enriched in the gut microbiome of patients
13 145 with RA and correlate positively with disease markers e.g. immunoglobulins IgA and IgG ,
14 146 while other taxa like *Haemophilus* spp. and *Faecalibacterium* spp. are typically found at lower
15 147 abundances in patients with RA compared to healthy individuals[13, 15, 16]. Alterations of
16 148 the gut microbiome may therefore have an important impact on RA pathophysiology[12].
17 149

18 150 PD affects 0.4-2% of the population above 65 years worldwide and is the second most
19 151 common progressive neurodegenerative disease with men being 1.5 times more likely to be
20 152 affected than women[17]. The cardinal symptoms are motor deficiencies such as tremor and
21 153 rigidity, but also include a wide range of non-motor symptoms, such as hyposmia, depression,
22 154 insomnia or cognitive impairment, severely impacting patients' quality of life[18].
23 155 Aggregations of the protein α -synuclein in the dopaminergic substantia nigra represent the
24 156 main neuropathological manifestations[19]. Recent literature suggests that
25 157 neuroinflammation plays a key role in early phases of PD-like neurodegeneration, with
26 158 microglia as the main cellular effectors[20]. Dysbiosis of the gut microbiome has been
27 159 associated with the characteristic motor deficits and pathophysiologic changes in the enteric
28 160 and central nervous systems in animal studies. Increased relative abundances of the genera
29 161 *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, and *Methanobrevibacter* and decreased
30 162 abundances in *Faecalibacterium* and *Roseburia* have been reported[21, 22]. The PD-
31 163 associated loss of dopaminergic neurons involves mechanisms of inflammatory and
32 164 autoimmune responses. The majority of patients with PD suffer from gastrointestinal
33 165 symptoms, e.g. constipation and irritable bowel syndrome (IBS) -like symptoms[23]. Recently
34 166 it has been suggested that the gut-brain axis, e.g. by-products produced by the gut
35 167 microbiome, may contribute to the production of α -synuclein aggregates in the enteric
36 168 nervous system[24]. In addition, increased intestinal permeability[25] and enteric
37 169 inflammation occur in PD and substantiate a role of peripheral inflammation in the initiation
38 170 and the progression of the disease[26].
39 171

40 172 One factor with known major impact on the gut microbiome and on chronic diseases is diet[7].
41 173 Dietary approaches, including fasting, have been used as therapeutic interventions at least
42 174 since the 5th century BCE[27, 28]. However, the available evidence from robust comparable
43 175 clinical studies is lacking. While PF of 7–10 days has been associated with improvements in
44 176 RA[29, 30], the mechanism behind the decrease in inflammatory processes during caloric
45 177 deprivation remains unclear. The improvements can typically only be maintained for a limited
46 178 period of time, and the symptoms can reappear after reintroduction of the patients' standard
47 179 diet. In mouse models of PD, intermittent fasting (IF) has led to several improvements
48 180 including decreased excitotoxicity, reduced neurodegeneration and protection against
49 181 autonomic dysfunction, motor and cognitive decline[31].

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3 182 IF and PF may have potent immunomodulatory effects which are partially mediated by the
4 183 gut microbiome and the fasting induced alterations of the latter[32]. These microbial shifts
5 184 include upregulation of *Akkermansia muciniphila*, *Bacteroides fragilis*, other *Bacteroides* spp.,
6 185 Proteobacteria, and butyric acid producing *Lachnospiraceae*, but also *Odoribacter*, which is
7 186 negatively associated with blood pressure[33, 34]. Interestingly, an overall decrease of the
8 187 Firmicutes/Bacteroidetes ratio could be observed, a high ratio is commonly associated with
9 188 several pathologies, including RA [35].
10
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12
13 190 To our knowledge no clinical trials have been investigating the connection between IF or PF
14 191 and PD in humans so far[31]. Our study aims to elucidate the causal relationship between the
15 192 gut microbiome and the immune system. To do so, we will use analyses of the molecular basis
16 193 of human-microbiome interactions enabled by high throughput methodologies such as the
17 194 combination of metagenomics, metatranscriptomics and metaproteomics. Moreover, we are
18 195 aiming at identifying new genes, proteins, metabolites and host pathways facilitating the
19 196 development of novel diagnostic and therapeutic tools[36, 37].
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22 23 24 198 **Methods and Analysis (2256)**

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26 199

27 28 200 **Study objectives**

29 201 The first objective of the study is to define specific gut microbiome-derived molecules in RA
30 202 and PD, compared to healthy individuals, and relate this information to the
31 203 immunophenotypes of the individuals. The second objective is to identify and track common
32 204 and disease-specific molecular signatures to predict the outcome of a gut microbiome-
33 205 targeted therapeutic intervention, here fasting, on inflammation-driven symptoms in RA and
34 206 PD. The third objective of the study is to identify and validate microbiome-derived effector
35 207 molecules which downregulate pro-inflammatory innate and adaptive immune pathways.
36
37 208

38 39 40 209 **Study design**

41 210 The ExpoBiome cohort consists of 180 adult individuals, meeting the exclusion and inclusion
42 211 criteria (Table 1), for the cross-sectional study (objectives 1 and 3) and 60 adult individuals for
43 212 the longitudinal study (objectives 2 and 3). There are five different arms in total: (1) RA –
44 213 cross-sectional arm [60 patients], (2) PD – cross-sectional arm [60 patients], and (3) healthy
45 214 controls – cross-sectional arm [60 patients], (4) RA – longitudinal arm [30 patients], (5) PD –
46 215 longitudinal arm [30 patients] (**Error! Reference source not found.**).
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48 216

49 217 At the first visit (T0), patients answer several questionnaires, and blood, urine, saliva and
50 218 stool samples are obtained (Table 2).

51 219 The longitudinal arms (1) and (2) undergo a 5–7-day PF with a dietary energy supply of max.
52 220 350-400 kcal per day with vegetable or grain broths as well as fresh vegetable juices[28, 33].
53 221 After the PF, the longitudinal arms follow a dietary regimen including the concept of TRE for
54 222 a period of 12 months following the 16:8 pattern[38]. This means that food intake is allowed
55 223 ad libitum for 8 h, followed by 16 h of fasting where no food should be consumed. The intake
56 224 of non-caloric beverages, e.g. water, unsweetened tea or coffee is, however, allowed. The
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225 participants attend one follow-up visit (T2) during the PF and 9 follow-up visits during the 12
 226 months of TRE (**Error! Reference source not found.**).

228 Patient and Public Involvement

229 Feedback of patients during former clinical trials at the study centre in Berlin was integrated
 230 in the planning and design of the fasting intervention of this study. Patients are not involved
 231 in the conduct, reporting, or dissemination plans of this research.

233 Recruitment and randomisation

234 Patients are recruited by the specialised sites via different sources, e.g. by direct referral from
 235 either a physician at the Immanuel Hospital Berlin and the outpatient department of the
 236 Institute of Social Medicine, Epidemiology and Health Economics at Charité-
 237 Universitätsmedizin Berlin, or the Paracelsus-Elena Clinic in Kassel, or by non-personal
 238 advertising strategies (e.g. flyers or social media).

239 Participants meeting all the inclusion and no exclusion criteria (Table 1) are assigned to their
 240 respective groups (RA, PD or healthy control) (**Error! Reference source not found.**) for the
 241 cross-sectional study after written informed consent. Half of the patients from the RA group
 242 and half of the patients from the PD group is selected to take part in the longitudinal part of
 243 the study, including the fasting intervention according to their availability for all 11 visits and
 244 their willingness to follow TRE over 12 months. This study is an open-label trial, as blinding is
 245 not feasible in fasting interventions.

246
 247

Table 1: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> • age 18-79 • one of the following diagnoses: rheumatoid arthritis (first diagnosis >6 weeks ago), Parkinson's disease OR healthy volunteer • control ("healthy") individuals must be without any evidence of active known or treated RA, without any evidence of active, known or treated central nervous system disease, and without a known family history of idiopathic PD • control individuals should match the RA or PD individuals as closely as possible (sex, age, education) • present written declaration of consent • ability to understand the patient information and willingness to sign the consent form 	<ul style="list-style-type: none"> • gout or proven bacterial arthritis • participation in another study • existing/current eating disorder (bulimia nervosa, anorexia nervosa) within the past 5 years • severe internal disease (e.g. kidney deficiency with creatinine > 2mg/dl) • existing vegan diet or fasting during the last six months • presence or suspicion of atypical PD (e.g. early dementia, early autonomous dysfunction) • diagnosis of chronic inflammatory bowel diseases, celiac disease or colorectal cancer according to the guidelines of the German Society of Gastroenterology • use of anti-psychotic drugs • antibiotic use during the previous 12 months

<ul style="list-style-type: none"> • consent to specimen collection and specimen use 	<ul style="list-style-type: none"> • start of novel therapy with disease-modifying anti-rheumatic drugs • pregnancy or breastfeeding women • contraindication for additional blood draws (e.g. haemoglobin <10) • BMI < 18.5 • Psychiatric illness that limits understanding of the examination protocol (unable to consent)
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249 **Fasting dietary counselling**

250 The fasting group is closely monitored by nutritionists trained in fasting therapy, backed up
 251 by physicians experienced in fasting, from the Charité – Universitätsmedizin Berlin and the
 252 Paracelsus-Elena Clinic to ensure a uniform implementation of the fasting guidelines and the
 253 well-being of the study participants. The monitoring consists of several in person and virtual
 254 meetings which held individually or in group settings. Five meetings including the visits T0 and
 255 T2 during the fasting week as well as a group meeting after PF to ensure a well-managed start
 256 to the TRE phase take place. Group sessions are standardised using a pre-set deck of slides to
 257 be discussed during the group meetings with only minor disease-related differences between
 258 the PD and RA groups. All longitudinal participants receive a study-specific script with
 259 information on fasting procedures.

260

261 **Medication**

262 The medical treatments of the patients are monitored and documented with every clinical
 263 visit. The fasting intervention might necessitate temporary adjustments of several
 264 medications e.g. anti-diabetic and anti-hypertensive drugs [28].

265

266 **Data collection**

267 Sample and data collection is performed at the two clinical sites, Charité – Universitätsmedizin
 268 Berlin and Paracelsus-Elena Clinic (Table 2).

269

270 *Table 2: Sampling procedures.*

a) Biochemical samples and procedures

Blood (123 mL at T0, 23 mL at T2-T11)
Stool collection (2 mL at T0 and T3-T11)
Saliva collection (3.5 mL at T0-T11)
Midstream urine (50 mL at T0 -T11)

271

b) Questionnaires

Disease specific
PD:

-
- Disease Activity Score (DAS-28) [39]
 - Parkinson's Disease Sleep Scale-2 (PDSS-2) [40]
 - Parkinson's Disease Questionnaire-39 (PDQ-39)[41]
 - Simplified Disease Index Score (SDAI) [42]
 - Funktionsfragebogen Hannover (FFbH-R) [43]
 - Movement Disorder Society Unified PD Rating Scale (MDS-UPDRS)[44]
 - Non-Motor Symptoms Questionnaire (NMSQ)[45]
 - Non-Motor Symptoms Scale (NMSS)[46]

RA:

- Disease Activity Score (DAS-28) [42]
- Non-Motor Symptoms Questionnaire (NMSQ) [45]
- Funktionsfragebogen Hannover (FFbH-R) [43]

Dietary behaviour and lifestyle

- Fasting experience, expectation, and intervention
- Lifestyle
- 24H-Food-recall
- Food Frequency Questionnaire (FFQ)

General health and well-being

- Health Assessment Questionnaire (HAQ)[47]
 - Bristol Stool Scale[48]
 - Quality of Life questionnaire (WHO-5)[49]
 - Hospital Anxiety and Depression Scale (HADS)[50]
 - Profile of Mood States[51]
-

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273

274 Anthropometric data and questionnaires

275 The electronic data capture system REDCap[52], a secure web-based application, is used to
 276 record all individual specific data. All data is stored on a secure server infrastructure at the
 277 host institution in Luxembourg. Weight, height, body mass index (BMI), heart rate and blood
 278 pressure in sitting and standing position as well as waist-hip-ratio is determined at every visit.

279 Dietary behaviour, sociodemographic measurements (age, sex, education level, employment
 280 status, marital status), family history, current and previous illness and co-morbidities, and
 281 current medications, as well as disease-specific data, questionnaires about the well-being of
 282 the patients and data on the behavioural factors are collected at baseline, T6 (week 3), T9
 283 (month 6) and T11 (month 12) (Table 2). Questionnaires (24h-Food Recall, Bristol Stool Scale)
 284 are answered at all visits by the study participants. Data storage, analysis and exchange are
 285 done only in pseudonymised fashion. The nutritional data is analysed using the Nutrilog 3.20
 286 software (Nutrilog SAS, Marans).

288 Blood samples and parameters

289 Blood samples are collected at each visit, and immediately used for peripheral blood
 290 mononuclear cell (PBMC) isolation (T0), analysis by the study laboratory and centrifugation
 291 to freeze plasma samples at -80°C (T0-T11). A clinical standard laboratory report is generated
 292 after every visit for each study participant (Table 3). In addition to the routine blood
 293 parameters, anti-citrullinated protein antibody (ACPA), zonulin, fatty-acid binding protein 2
 294 (FABP2), and calprotectin are measured. Aliquots are securely stored to account for novel
 295 observations and testing of hypotheses.

296
 297 *Table 3: Routine blood parameters measured at each timepoint (T0 for cross-sectional study, T0-T11 for longitudinal study)*

Haematology - EDTA-blood	Clinical Chemistry - Serum
Basophils, %	Albumin
Basophils, abs.	ALT, 37°C
Eosinophils, %	Alkaline Phosphatase, 37°C
Eosinophils, abs.	AST, 37°C
Erythrocytes	Bilirubin, total
Haematocrit	Cholinesterase
Haemoglobin	Cholesterol
HbA1c	Creatinine
Leucocytes	hs-CRP
Lymphocytes, %	Glucose, serum
Lymphocytes, abs.	Gamma-GT, 37°C
MCH	HDL-Cholesterol
MCHC	LDL-Cholesterol
MCV	Potassium
Monocytes, %	Sodium
Monocytes, abs.	Total Protein
Neutrophils, %	Triglycerides

Neutrophils, abs.	Uric Acid
Platelets	Urea/BUN
RDW	Proteins - Serum
Reticulocytes	Rheumatoid factor H 35.9
Reticulocytes	Hormones - Serum
Reticulocytes, abs.	Insulin
	TSH (basal)

298

299 Stool, urine and saliva samples

300 The samples listed in Table 2 are collected at each visit, except for stool samples on T2 (fasting
 301 week) and immediately frozen and stored at -80°C. Stool characteristics are recorded at the
 302 time of the sampling. Faecal samples represent the main sample type for resolving the
 303 dynamic processes driven by microbiome in the gut. Also, as the gut microbiome is prone to
 304 diurnal fluctuations, the stool samples are, as far as possible, collected in the morning.

305

306 Methods applied to samples

307

308 Biomolecular extractions

309 The collected stool samples undergo a biomolecular extraction procedure to allow isolation
 310 of concomitant DNA, RNA, proteins, peptides and metabolites from single, unique faecal
 311 water samples[53, 54]. Faecal water is recovered following centrifugation and filtration, at
 312 low-speed or low-flow, respectively, to avoid cell lysis. Nucleic acids are preserved by the
 313 addition of ribonuclease inhibitors and isolated by silica-column-based techniques. This
 314 protocol involves the use of a robotic platform, ensuring a higher level of standardisation and
 315 reproducibility[2].

316

317 Coupled metagenomic and metatranscriptomic analyses

318 Prior to sequencing library preparation, internal standards are introduced to obtain
 319 quantitative sequencing data[55]. Contamination-free metagenomic (MG) and
 320 metatranscriptomic (MT) data is generated, processed and analysed using the Integrated
 321 Meta-omics Pipeline (IMP)[36], which incorporates pre-processing, assembly, gene
 322 annotation, mapping of reads, single nucleotide polymorphism calling, data normalisation as
 323 well as analyses of community structure and function in a fully reproducible software
 324 framework based on Docker. The MG and MT data is specifically screened for enrichments in
 325 genes and pathways with known immunogenic properties[56]. The extracellular biomolecules
 326 are linked to specific microbial populations based on the intracellular metagenomic data [57].
 327 In addition, the sequencing data is mapped against genomes of food components[58]. The
 328 quantitative data is also related to microbial population sizes to determine the contribution
 329 of the resolved microbial populations in stool to the extracellular DNA and RNA
 330 complements[59].

331

332 Metaproteomics

333 For the metaproteomic analyses, filtration is used to separate extracellular peptides from the
334 obtained (poly)peptides. The resulting smaller fractions are then desalted and analysed
335 without proteolytic digestion via liquid chromatography (LC) and mass spectrometry (MS) on
336 an EasyNano-LC coupled online to a QExactive-Plus mass spectrometer (ThermoScientific,
337 USA). The identification of ribosomal peptides is done with an integrated catalogue of MG
338 and MT data, while the non-ribosomal peptides are identified using different tools, i.e.,
339 MyriMatch, DirecTag as well as CycloBranch[36, 60, 61]. The metaproteomic data also allows
340 identification of extracellular (poly)peptides with possible pathogenic functions including
341 protein misfolding and molecular mimicry[62, 63].

342

343 Metabolomics

344 Metabolomic data is analysed using a combination of targeted and untargeted approaches
345 [54, 58, 64]. This highlights the major metabolite classes produced by the gut microbiome
346 with an effect on human physiology including organic acids, short-chain fatty acids, lipids,
347 branched-chain fatty acids, branched-chain amino acids, vitamins, bile acids and
348 neurotransmitters. Besides external compound calibration series for quantification and
349 quality control samples to ensure data normalisation and data acquisition quality assessment,
350 the metabolite extraction fluid is fortified with multiple internal standards to improve method
351 precision and accuracy[65, 66]. The data is compared to in-house databases and public mass
352 spectral libraries to identify known metabolites. The metabolomic data complements the
353 metagenomic and metatranscriptomic data and thus allows further establishments of
354 conclusive links to metabolic properties in the gut.

355

356 Deep immune profiling

357 Deep immune profiling is done using a recently established and optimised panel of metal-
358 labelled antibodies together with cytometry coupled to mass spectrometry (MS), the Maxpar
359 Direct Immune Profiling System (MDIPA). This approach allows the simultaneous
360 quantification of 38 parameters on single cells. Whole blood is stained with the MDIPA kit and
361 stabilised with Proteomic stabiliser Prot-1 (501351694, Smart Tube Inc., Las Vegas) before
362 storage at -80°C. The quantified immune cells included in the MDIPA panel are CD3+, CD4+,
363 CD8+, monocytes, dendritic cells, granulocytes, MAIT, T cells, NK and B cells[67]. Cytokine
364 expression profiles is analysed on blood plasma using the Human Luminex performance
365 Cytokine Panel (R&D Systems Europe, Abingdon), measuring CCL3, CCL4, CCL5, GM-CSF, IL-
366 1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-18, IL-21, IL-27, IL-33, IFN- β ,
367 Galectin-1, IFN- γ and TNF- α [56].

368

369 Gut-on-a-chip models

370 PBMCs isolated from T0 blood samples are co-cultured with gut-derived microbes under
371 physiologically representative conditions using the gut-on-a-chip model HuMiX[68]. This
372 model of the human gastrointestinal interface allows the investigation of the interactions
373 between immune, epithelial and bacterial cells and specifically the response to fasting in
374 personalised in vitro models.

375

376 The Expobiome Map

377 The Expobiome Map (<https://expobiome.lcsb.uni.lu>) illustrates the diverse complex of
378 microbial immunogenic molecules, including nucleic acids, (poly)peptides, structural
379 molecules, and metabolites. The interactions between this “expobiome” and human immune
380 pathways are encoded in the context of chronic diseases[1]. The ExpoBiome Map is visualised
381 using the MINERVA Platform[69]. Clicking on different elements on the map reveals factors
382 they affect and are affected by, allowing an easier navigation through the complex
383 relationships between individual microbiome components in relation to human disease. The
384 multi-omics data generated in the present study will be integrated with the Map.

385

386 Exploratory analysis of novel host-microbiome interactions

387 Unknown non-ribosomal peptides or metabolite features are associated through correlation
388 with transcripts, proteins and metabolites. Extracellular DNA fragments, as well as transcripts,
389 proteins and ribosomal peptides are linked to their genomic context by using IMP[36]. The
390 data generated by the project will be connected and collated to existing, publicly available
391 datasets.

392

393 Outcome parameters

394 Primary Outcome

395 The primary endpoint of the study is the characterisation of the gut microbiome. The
396 evaluation includes both between-group and within-group differences in the longitudinal
397 study arms with the fasting intervention.

398

399 Secondary Outcome Measures

400 Secondary outcomes include the identification of common and disease-specific molecular
401 signatures and the characterisation of microbiome-derived effector molecules impacting the
402 innate and adaptive immune pathways. Furthermore, several additional parameters
403 mentioned in *Anthropometric data and questionnaires* are assessed over a period of 12
404 months.

405

406 Sample size and power calculation

407 A power calculation using pilot metatranscriptomic data based on faecal extracellular RNA
408 samples was performed to determine the number of subjects to be recruited for the
409 ExpoBiome project. The obtained relative abundances of genera were used for the calculation
410 of the required sample size per group. The power calculation was based on the algorithm as
411 described by Tusher, Tibshirani, and Chu[70]. To achieve a power of 90% (at $\alpha = 0.05$), a total
412 of 50 individuals per group (RA, PD, healthy controls) must be analysed. Considering any
413 possible dropouts, 20% additional subjects are recruited, resulting in a total number of 180
414 individuals, i.e., 60 per group.

415

416 **Adverse events**

417 There are no major risks expected for participants. Minor common adverse effects of PF might
418 include headaches, nausea, insomnia, back pain, dyspepsia and fatigue[71]. Any occurring
419 adverse events are recorded at each visit in REDCap[52]. Serious adverse events are
420 communicated to the study coordinator and principal investigator within 24 h of their report.
421

422 **Data management, monitoring, analysis and evaluation of data**

423 The study participants receive a study ID (pseudonym) which is used for all collected data.
424 Self-administered questionnaires are directly recorded in REDCap. Participant files are kept
425 for at least 10 years at the respective clinical sites.

426 Weekly meetings between the study team, the different clinical partners and the principal
427 investigator, ensure a close monitoring of the data. Any occurring adverse events or other
428 issues are thus handled immediately.

429 Different statistical tests are performed according to the nature of the data. A premature
430 termination of the study is not envisaged; therefore, no interim analysis is done. Different
431 correlation measures are applied, including Spearman correlation, mutual information on
432 discretised data, distance correlation, maximum information criterion, local similarity analysis
433 and the bioenv approach. Comparison across all omic levels allows identification of common
434 and disease-specific signatures. Multivariate machine learning is used to link different data
435 features to observed patterns.

436 The longitudinal part of the study continues for a period of 12 months. After finalisation of
437 this period, there is no follow-up of the participants. Interesting findings will be further
438 validated using the existing sample set and analyses may be performed on additionally
439 collected samples.

440 **Discussion (869)**

441 The impact and importance of the gut microbiome on human physiology and its potential
442 modifications by nutrition and dietary patterns, have been underestimated for
443 centuries[72].

444 More recently, physical as well as mental well-being has been related to the gut microbiome
445 composition and new associations to human health are emerging almost every day. The gut
446 microbiome is responsible for the fermentation of indigestible food components, the
447 synthesis of numerous metabolites including vitamins, the removal of toxic compounds and
448 pathogens, the maintenance and strengthening of the intestinal barrier and immune system
449 function amongst others[21].

450 Globally we observe a gradual shift to a Western diet, typically low in micronutrients, mono-
451 and polyunsaturated fats and linoleic acid but high in saturated fat, refined carbohydrates,
452 salt and processed food. This shift is associated with an emergence of non-communicable
453 diseases (NCDs) such as obesity, cardiovascular diseases, type 2 diabetes mellitus, cancer,
454 liver and gastrointestinal diseases, bone and joint diseases, and degenerative diseases of the
455 brain[73]. Not surprisingly, obesity and diabetes were some of the first diseases to be linked
456 to the gut microbiome composition[74]. Psychological disorders like anxiety and depression
457 have also been associated with the gut microbiome[75], emphasizing the importance of the
458 bacterial community in various facets of health.
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3 460 Fasting has been used as an intervention to promote health or to heal diseases since the
4 461 beginning of civilisations and has spread independently among different regions, cultures and
5 462 religions worldwide[76]. It is believed to have already been established as a treatment
6 463 method by Hippocrates in the 5th century BCE and has been used ever since by numerous
7 464 medical schools to treat acute and chronic diseases[27, 77]. Various practices of caloric
8 465 restriction through fasting have repeatedly shown remarkable health benefits[78, 79].
9 466 Maifeld *et al.* for instance found that a 5-day fast followed by a modified Dietary Approach to
10 467 Stop Hypertension (DASH), with additional emphasis on plant-based and Mediterranean
11 468 diets, reduced systolic blood pressure, BMI, and the need for antihypertensive medications
12 469 at three months post intervention compared to DASH alone. Bacterial taxa and genes
13 470 associated with short-chain fatty acid production were altered in the gut, e.g. many Clostridial
14 471 Firmicutes shifted significantly in abundance, with an initial decrease in butyrate producers
15 472 such as *Faecalibacterium prausnitzii* [34].
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20 474 Furthermore, Choi *et al.* demonstrated that cycles of a fasting-mimicking diet suppress
21 475 autoimmunity and stimulate remyelination via oligodendrocyte regeneration in a murine
22 476 experimental autoimmune encephalomyelitis (EAE) model[80]. Jordan *et al.* described a
23 477 reduction in monocyte metabolic and inflammatory activity after a short-term fast and
24 478 conclude that fasting attenuates chronic inflammatory diseases without compromising
25 479 monocyte capacity for mobilisation during acute infectious inflammation and tissue
26 480 repair[81].
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29 481

30 482 Diet as a tool to prevent disease, considering both the quantity and the quality of food,
31 483 remains underappreciated in routine clinical practice as well as in research. Reasons may
32 484 include missing standardised therapeutic protocols, the interindividual variability in the
33 485 response to fasting, lack of knowledge about possible adverse effects, and difficulties in the
34 486 interpretation of underlying mechanisms seen in clinical trials, but also in the comparably low
35 487 potential for achieving economic revenue or scientific impact[8].
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37 488

38
39 489 Modern experimental approaches and computational integration allow a multi-layer analysis
40 490 of digestive processes in low caloric settings including the gut microbiome[58]. These
41 491 technological developments also permit a closer investigation of the link between the
42 492 immune system and severe caloric restriction.
43
44 493

45 494 Fasting-induced changes in the gut microbiome are associated with host energy
46 495 metabolism[33], with a shift in the gut microbiome after as little as 24h[72]. Although the
47 496 changes in gut microbiome composition induced by a 10-day PF of healthy individuals return
48 497 to baseline after 3 months, the resilience of the initial gut microbiome composition might not
49 498 be the same in unhealthy individuals[33]. Currently available knowledge is insufficient to
50 499 define a healthy gut microbiome due to high interindividual variability. However, several
51 500 characteristics such as increased diversity in microbial taxa and their gene richness, a high
52 501 amount of butyrate producers and resilience of the microbial community are often
53 502 considered as beneficial[82]. Thus, a dysbiotic gut microbiome is likely to be less resilient and
54 503 therefore more susceptible to dietary interventions, enabling a possible reversion to a healthy
55 504 status[72].
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3 506 A potential mechanism underlying the observed beneficial effects induced by dietary
4 507 interventions might be a direct gut microbiome-immune system interaction by pattern
5 508 recognition. The microbiome can regulate the intestinal innate immune system by modulating
6 509 toll-like receptor (TLR) expression on immunosensor cell surface through microbe-associated
7 510 molecular patterns (MAMPs), which can consequently trigger cytokine production and up-
8 511 regulation of molecules on antigen presenting cells, leading to activation of T cells[83].
9 512 Therefore, a change in gut microbiome composition can lead to different outcomes in
10 513 immune signaling pathways and either favor or suppress inflammation and autoimmunity.
11
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13
14 515 The current evidence highlights the immensely important connection between the
15 516 environment and health, including dietary habits, microbiome and the immune system.
16 517 Combining the newly generated data of this study with knowledge from disciplines such as
17 518 nutrigenomics could potentially lead to more extended and deepened utilisation of
18 519 personalised nutrition. Taking the gut microbiome into account as a key factor in disease for
19 520 nutritional recommendations might revolutionise the current guidelines and allow
20 521 substantially more efficient proposals. These approaches might eventually be broadly
21 522 applicable and constitute an efficient antidote against the multiple burden of malnutrition
22 523 and over-alimentation and the prominent NCDs.
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27 524 **Trial status**

28 525 The recruitment for the ExpoBiome study started in April 2021 and is currently ongoing. All
29 526 study participants should be recruited by the end of 2022.
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31 527

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36
37 531

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48 540 - Initial draft of the manuscript and coordinated the editing process: Bérénice Hansen
49 541 - Contributed equally with edits, comments and feedback, read and approved the final
50 542 manuscript: all authors
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59
60

547 Competing interests statement

548 None declared.

549 Supplements

550 We used the SPIRIT checklist when writing our report[84].

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36 755 Figure Legends

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39 756 Figure 1. Study design. This figure illustrates the study design with five different arms in
40 757 total, two of which continue with the longitudinal part of the study. Visits take place at the
41 758 clinical sites at each timepoint and include the collection of the displayed samples. This
42 759 image was generated using Biorender software (<http://www.biorender.com>). T, timepoint;
43 760 W, week; D, day; M, month.
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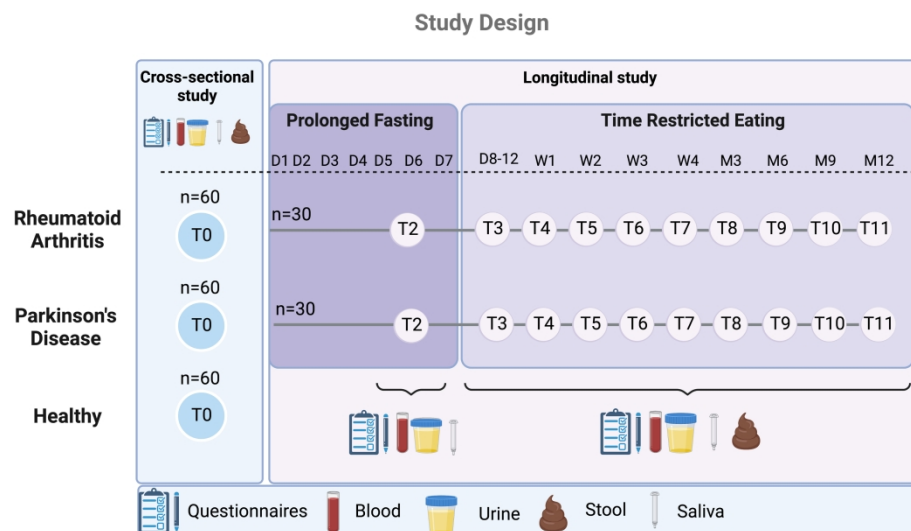


Figure 1. Study design. This figure illustrates the study design with five different arms in total, two of which continue with the longitudinal part of the study. Visits take place at the clinical sites at each timepoint and include the collection of the displayed samples. This image was generated using Biorender software (<http://www.biorender.com>). T, timepoint; W, week; D, day; M, month.

279x177mm (300 x 300 DPI)

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRIT reporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Gøtzsche PC, Altman DG, Mann H, Berlin J, Dickersin K, Hróbjartsson A, Schulz KF, Parulekar WR, Krleža-Jerić K, Laupacis A, Moher D. SPIRIT 2013 Explanation and Elaboration: Guidance for protocols of clinical trials. *BMJ*. 2013;346:e7586

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	n/a
Protocol version	#3	Date and version identifier	n/a
Funding	#4	Sources and types of financial, material, and other support	14
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
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7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	1, 14
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
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16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	1, 14
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
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24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	3
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
31				
32	Background and	#6b	Explanation for choice of comparators	2
33	rationale: choice of			
34	comparators			
35				
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37	Objectives	#7	Specific objectives or hypotheses	5
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	5
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
50				
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52				
53	Study setting	#9	Description of study settings (eg, community clinic, academic	6
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
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1	Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
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6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	5
7	description			
8				
9				
10	Interventions:	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	5
11	modifications			
12				
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14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	7
16	adherence			
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20	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	7
21	concomitant care			
22				
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24	Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	8
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34	Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	5
35				
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40	Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	11
41				
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45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	5
46				
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48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
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53				
54	Allocation: sequence	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be	n/a
55	generation			
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provided in a separate document that is unavailable to those who enrol participants or assign interventions

Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	n/a
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	n/a
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	n/a
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	n/a
Methods: Data collection, management, and analysis			
Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	8
Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	9
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	12
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	12

1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	12
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	12
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
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10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	12
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
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22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	n/a
23	interim analysis		including who will have access to these interim results and make	
24			the final decision to terminate the trial	
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26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	12
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
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33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	n/a
34			whether the process will be independent from investigators and	
35			the sponsor	
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38	Ethics and			
39	dissemination			
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42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	2
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	2
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
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53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	6
54			participants or authorised surrogates, and how (see Item 32)	
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1	Consent or assent:	#26b	Additional consent provisions for collection and use of	6
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
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6	Confidentiality	#27	How personal information about potential and enrolled	12
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
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11	Declaration of interests	#28	Financial and other competing interests for principal investigators	14
12			for the overall trial and each study site	
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15	Data access	#29	Statement of who will have access to the final trial dataset, and	12
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
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20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	n/a
21	care		compensation to those who suffer harm from trial participation	
22				
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24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	2
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
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33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	14
34	authorship		professional writers	
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36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
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41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	6
44	materials		participants and authorised surrogates	
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46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	7
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
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BMJ Open

Protocol for a multicentre cross-sectional, longitudinal clinical trial in rheumatoid arthritis and Parkinson's disease patients analysing the relation between the gut microbiome, fasting and immune status (ExpoBiome)

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2022-071380.R1
Article Type:	Protocol
Date Submitted by the Author:	31-May-2023
Complete List of Authors:	<p>Hansen, Bérénice; LCSB Laczny, Cédric C.; LCSB Aho, Velma T.E.; LCSB Frachet-Bour, Audrey; LCSB Habier, Janine; LCSB Ostaszewski, Marek; LCSB Michalsen, Andreas; Charité Universitätsmedizin Berlin, Institute for Social Medicine, Epidemiology and Health Economics; Immanuel Hospital Berlin-Wannsee Branch, Department of Internal and Integrative Medicine Hanslian, Etienne; Charite Universitätsmedizin Berlin, Institute for Social Medicine, Epidemiology and Health Economics; Immanuel Hospital Berlin-Wannsee Branch Koppold-Liebscher, Daniela; Charité Universitätsmedizin Berlin, Institute for Social Medicine, Epidemiology and Health Economics; Immanuel Hospital Berlin-Wannsee Branch Hartmann, Anika; Charité Universitätsmedizin Berlin, Institute of Social Medicine, Epidemiology and Health Economics; Charité Universitätsmedizin Berlin, Department of Dermatology, Venereology and Allergology Steckhan, Nico; Charité Universitätsmedizin Berlin, Institute for Social Medicine, Epidemiology and Health Economics; University of Potsdam, Digital Health - Connected Healthcare, Hasso Plattner Institute Mollenhauer, Brit; University Medical Center Göttingen; Paracelsus-Kliniken Deutschland GmbH Schade, Sebastian; University Medical Center Göttingen; Paracelsus-Kliniken Deutschland GmbH, Roomp, Kirsten; LCSB Schneider, Jochen; LCSB; Saarland University Hospital and Saarland University Faculty of Medicine, Department of Internal Medicine and Psychiatry Wilmes, Paul; LCSB; University of Luxembourg, Department of Life Sciences and Medicine</p>
Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Immunology (including allergy), Rheumatology, Pharmacology and therapeutics, Neurology, Evidence based practice

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Keywords:	IMMUNOLOGY, Rheumatology < INTERNAL MEDICINE, MICROBIOLOGY, Parkinson-s disease < NEUROLOGY, NUTRITION & DIETETICS, Clinical trials < THERAPEUTICS

SCHOLARONE™
Manuscripts

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4 1 **Protocol for a multicentre cross-sectional, longitudinal clinical trial in**
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6 2 **rheumatoid arthritis and Parkinson's disease patients analysing the**
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8 3 **relation between the gut microbiome, fasting and immune status**
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10 **(ExpoBiome)**
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14 6 Bérénice Hansen¹, Cédric C. Laczny¹, Velma T.E. Aho¹, Audrey Frachet-Bour¹, Janine Habier¹, Marek
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59 51 Word count: 3646
60

Abstract

Introduction

Chronic inflammatory diseases like rheumatoid arthritis (RA) and neurodegenerative disorders like Parkinson's disease (PD) have recently been associated with a decreased diversity in the gut microbiome, emerging as key driver of various diseases. The specific interactions between gut-borne microorganisms and host pathophysiology remain largely unclear. The microbiome can be modulated by interventions comprising nutrition.

The aim of our clinical study is to (1) examine effects of prolonged fasting and time-restricted eating (TRE) on the outcome parameters and the immunophenotypes of RA and PD with (2) special consideration of microbial taxa and molecules associated with changes expected in (1) and (3) identify factors impacting the disease course and treatment by in depth screening of microorganisms and molecules in personalised HuMiX gut-on-chip models, to find novel targets for anti-inflammatory therapy.

Methods and Analysis

This trial is an open-label, multicentre, controlled clinical trial consisting of a cross-sectional and a longitudinal study. A total of 180 patients is recruited. For the cross-sectional study, 60 patients with PD, 60 patients with RA and 60 healthy controls are recruited at two different, specialized clinical sites. For the longitudinal part, 30 patients with PD and 30 patients with RA undergo 5-7 days of prolonged fasting (PF) followed by TRE (16:8) for a period of 12 months. One baseline visit takes place before the PF intervention and 10 follow-up visits will follow over a period of 12 months (April 2021 to November 2023).

Ethics and dissemination

Ethical approval was obtained to plan and conduct the trial from the institutional review board of the Charité-Universitätsmedizin Berlin (EA1/204/19), the ethics committee of the state medical association (Landesärztekammer) of Hessen (2021-2230-zvBO) and the Ethics Review Panel (ERP) of the University of Luxembourg (ERP 21-001-A ExpoBiome). The results of this study will be disseminated through peer-reviewed publications and scientific presentations.

Trial registration number at clinicaltrials.gov:

NCT04847011

Key words: Microbiome, fasting therapy, intermittent fasting, time restricted eating, chronic disease, rheumatoid arthritis, Parkinson's disease, nutrition, chronic diseases, ExpoBiome, inflammation, gut on a chip, HuMiX, immunophenotype, metagenomics, metatranscriptomics, metaproteomics, metabolomics

Strengths and limitations of the study

- The participants of the longitudinal study will be closely monitored for 12 months and routine blood parameters as well as anthropometric data and questionnaires will be precisely documented.

- 94 • This study will identify novel microbiome-derived common and disease-associated
95 molecules involved in immune system modulation in two major chronic diseases: RA
96 and PD.
- 97 • This study aims at also identifying novel targeted pathways to control chronic
98 inflammatory conditions in the future.
- 99 • A limitation is the heterogeneity of the cohorts regarding age and sex, which is due to
100 the prevalence of the diseases: RA is more common in women, while PD is more
101 common in men and has a later disease onset.
- 102 • A bias exists in choosing RA and PD as chronic disorders to study immunophenotypes
103 although generalisable results are targeted.
104

105 Introduction (1339)

106
107 The human microbiome is emerging as a key driver of various diseases through its complex of distinct
108 yet connected biomolecules (referred to as the “*expobiome*”)[1, 2]. The expobiome comprises a
109 diverse set of nucleic acids, polypeptides and metabolites which, in the gut alone, are present in
110 substantial concentrations[1]. However, the specific interactions between gut-borne microorganisms
111 and host (patho)physiology remain largely unknown. Although host genetics shape the composition
112 of the gut microbiome, the latter is particularly influenced by non-genetic factors such as lifestyle and
113 diet[3, 4]. Therefore, the microbiome is a plausible target to modify health outcomes.

114
115 Individuals suffering from chronic diseases, including autoimmune, metabolic, and neurodegenerative
116 diseases as well as cancer, often present alterations in their gut microbiome composition. These shifts
117 are typically characterised by an overgrowth of one or several microbial species with likely adverse
118 effects as well as a decrease in beneficial taxa[5]. Such imbalances are referred to as dysbiosis.
119 Although structural microbiome changes are clearly detectable, the mechanistic or functional
120 consequences of dysbiosis are still largely unknown. However, they may result in dysregulated
121 interactions with the immune system[6]. Considering the intricacy of the immune system, the
122 question arises whether the observed microbiome changes are cause or consequence of disease. This
123 implies that, in addition to the genetic predisposition of the host, the gut microbiome needs to be
124 considered a potential pathogenic factor or major driver of disease onset and course[3, 4].
125

126 RA and PD are two specific examples representing dysregulated microbiome-immune system
127 interactions [7, 8]. RA is a multifactorial, chronic and systemic autoimmune disease, primarily affecting
128 the lining of the synovial joints with a higher risk and younger age for disease onset in women and a
129 global prevalence of 1%[9, 10]. The exact disease pathogenesis is still unclear and no cure for RA
130 currently exists. In addition to the common local articular symptoms of RA, systemic comorbidities can
131 affect the vasculature, metabolism and bones[11]. Besides various environmental risk factors e.g.
132 smoking and a Western diet, the host microbiome is associated with the pathophysiology of the
133 disease[12]. The diversity of the gut microbiome has been reported to be decreased in individuals with
134 RA, compared with the general population, and is correlated with disease duration, activity and
135 autoantibody levels[13, 14]. Studies in murine models also report that autoimmune arthritis is strongly
136 attenuated under germ-free conditions[15]. The introduction of specific bacteria, e.g. segmented
137 filamentous bacteria, into germ-free animals or oral infection with *Porphyromonas gingivalis* drive
138 autoimmune arthritis through activation of T helper cells[15]. Several different taxa, including
139 *Prevotella copri*, *Lactobacillus* spp. and *Colinsella* spp. are enriched in the gut microbiome of patients
140 with RA and correlate positively with disease markers e.g. immunoglobulins IgA and IgG , while other
141 taxa like *Haemophilus* spp. and *Faecalibacterium* spp. are typically found at lower abundances in

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3 142 patients with RA compared to healthy individuals[13, 16, 17]. Alterations of the gut microbiome may
4 143 therefore have an important impact on RA pathophysiology[12].
5 144

6 145 PD affects 0.4-2% of the population over 65 years worldwide and is the second most common
7 146 progressive neurodegenerative disease with men being 1.5 times more likely to be affected than
8 147 women[18]. Cardinal symptoms are motor deficiencies such as tremor and rigidity, but also include a
9 148 wide range of non-motor symptoms, such as hyposmia, depression, insomnia or cognitive impairment,
10 149 severely impacting patients' quality of life[19]. Aggregations of the protein α -synuclein in the
11 150 dopaminergic substantia nigra represent the main neuropathological manifestations[20]. PD-
12 151 associated loss of dopaminergic neurons involves mechanisms of inflammatory and autoimmune
13 152 responses with microglial activity as a major driver [21].Dysbiosis of the gut microbiome has been
14 153 associated with the characteristic motor deficits and pathophysiological changes in the enteric and
15 154 central nervous systems in animal studies. Increased relative abundances of the genera *Akkermansia*,
16 155 *Bifidobacterium*, *Lactobacillus*, and *Methanobrevibacter* and decreased abundances in
17 156 *Faecalibacterium* and *Roseburia* have been reported[22, 23]. Two recently published clinical-trials
18 157 with prebiotic supplementation in PD observed a shift in gut microbiome composition, an increase in
19 158 short-chain fatty acids (SCFA) and a reduction in non-motor-symptoms [24, 25]. Most patients with
20 159 PD suffer from gastrointestinal symptoms such as constipation and irritable bowel syndrome (IBS) -
21 160 like symptoms[26]. The gut-brain axis, e.g. by-products produced by the gut microbiome, may
22 161 contribute to the production of α -synuclein aggregates in the enteric nervous system[27]. In addition,
23 162 increased intestinal permeability[28] as driver for enteric inflammation occur in PD and substantiate
24 163 a role of peripheral inflammation in the initiation and the progression of the disease[29].
25 164

26 165 One factor with known major impact on the gut microbiome and on chronic diseases is diet[7]. Dietary
27 166 approaches as fasting have already been used by Hippocrates in the the 5th century BCE and have been
28 167 applied ever since by numerous medical schools to treat acute and chronic diseases [30-32]. Various
29 168 practices of caloric restriction through fasting have repeatedly shown remarkable health benefits[33,
30 169 34]. Maifeld *et al.* found that a 5-day fast followed by a modified Dietary Approach to Stop
31 170 Hypertension (DASH), with additional emphasis on plant-based and Mediterranean diets, reduced
32 171 systolic blood pressure, BMI, and the need for antihypertensive medications at three months post
33 172 intervention compared with DASH alone [35].
34 173

35 174 Furthermore, Choi *et al.* demonstrated that cycles of a fasting-mimicking diet suppress autoimmunity
36 175 and stimulate remyelination via oligodendrocyte regeneration in a murine experimental autoimmune
37 176 encephalomyelitis (EAE) model[36]. Jordan *et al.* described a reduction in monocyte metabolic and
38 177 inflammatory activity after a short-term fast and conclude that fasting attenuates chronic
39 178 inflammatory diseases without compromising monocyte capacity for mobilisation during acute
40 179 infectious inflammation and tissue repair[37].
41 180

42 181 These improvements can, however, typically only be maintained for a limited period of time, and the
43 182 symptoms can reappear after reintroduction of the patients' standard diet. Hence, protocols to sustain
44 183 these beneficial effects are of utmost importance. In mouse models of PD, intermittent fasting (IF) has
45 184 led to several improvements including decreased excitotoxicity, reduced neurodegeneration and
46 185 protection against autonomic dysfunction, motor and cognitive decline[38].
47 186

48 187 IF and PF may have potent immunomodulatory effects which may partially be mediated by the gut
49 188 microbiome and the fasting induced alterations of the latter[39]. These microbial shifts include
50 189 upregulation of *Akkermansia muciniphila*, *Bacteroides fragilis*, other *Bacteroides* spp., Proteobacteria,
51 190 and butyric acid producing *Lachnospiraceae*, but also *Odoribacter*, which is negatively associated with
52 191 blood pressure[35, 40]. Interestingly, an overall decrease of the Firmicutes/Bacteroidetes ratio could
53 192 be observed, a high ratio is commonly associated with several pathologies, including RA [41].

193
194 A potential mechanism underlying the observed beneficial effects induced by dietary interventions
195 might be a direct gut microbiome-immune system interaction by pattern recognition. The microbiome
196 can regulate the intestinal innate immune system by modulating toll-like receptor (TLR) expression on
197 immunosensor cell surface through microbe-associated molecular patterns (MAMPs), which can
198 consequently trigger cytokine production and up-regulation of molecules on antigen presenting cells,
199 leading to activation of T cells[42]. Therefore, a change in gut microbiome composition can lead to
200 different outcomes in immune signalling pathways and either favour or suppress inflammation and
201 autoimmunity.

202
203 The impact and importance of the gut microbiome on human physiology and its potential
204 modifications by nutrition and dietary patterns, have been underestimated for centuries[43]. Reasons
205 may include missing standardised therapeutic protocols, the interindividual variability in the response
206 to fasting, lack of knowledge about possible adverse effects, and difficulties in the interpretation of
207 underlying mechanisms seen in clinical trials, but also in the comparably low potential for achieving
208 economic revenue or scientific impact[8].

209
210 Modern experimental approaches and computational integration allow a multi-layer analysis of
211 digestive processes in low caloric settings including the gut microbiome[44]. These technological
212 developments also permit a closer investigation of the link between the immune system and severe
213 caloric restriction.

214
215 To our knowledge no clinical trials have been investigating the connection between IF or PF and PD in
216 humans so far[38]. Our study aims to elucidate the causal relationship between the gut microbiome
217 and the immune system. To do so, we will use analyses of the molecular basis of human-microbiome
218 interactions enabled by high throughput methodologies such as the combination of metagenomics,
219 metatranscriptomics and metaproteomics. Moreover, we are aiming at identifying new genes,
220 proteins, metabolites, and host pathways facilitating the development of novel diagnostic and
221 therapeutic tools[45, 46].

222

223 **Methods and Analysis (2317)**

224

225 **Study objectives**

226 The first objective of the study is to define specific gut microbiome-derived molecules in RA and PD,
227 compared to healthy individuals, and relate this information to the immunophenotypes of the
228 individuals. The second objective is to identify and track common and disease-specific molecular
229 signatures to predict the outcome of a gut microbiome-targeted therapeutic intervention, here
230 fasting, on inflammation-driven symptoms in RA and PD. The third objective of the study is to identify
231 and validate microbiome-derived effector molecules which downregulate pro-inflammatory innate
232 and adaptive immune pathways.

233

234 **Study design**

235 The ExpoBiome cohort consists of 180 adult individuals, meeting the exclusion and inclusion criteria
236 (Table 1), for the cross-sectional study (objectives 1 and 3) and 60 adult individuals for the longitudinal
237 study (objectives 2 and 3). There are five different arms in total: (1) RA – cross-sectional arm [60
238 patients], (2) PD – cross-sectional arm [60 patients], and (3) healthy controls – cross-sectional arm [60
239 patients], (4) RA – longitudinal arm [30 patients], (5) PD – longitudinal arm [30 patients] (Figure1).

240

At the first visit (T0), patients answer several questionnaires, and blood, urine, saliva and stool samples are obtained (Table 2). The longitudinal arms (4) and (5) undergo a 5–7-day PF with a dietary energy supply of max. 350-400 kcal per day with vegetable or grain broths as well as fresh vegetable juices[31, 40]. After the PF, the longitudinal arms follow a dietary regimen including the concept of TRE for a period of 12 months following the 16:8 pattern[47]. This means that food intake is allowed ad libitum for 8 h, followed by 16 h of fasting where no food should be consumed. The intake of non-caloric beverages, e.g. water, unsweetened tea or coffee is, however, allowed. The participants attend one follow-up visit (T2) during the PF and 9 follow-up visits during the 12 months of TRE (Figure 1).

Patient and Public Involvement

Feedback of patients during former clinical trials at the study centre in Berlin was integrated in the planning and design of the fasting intervention of this study. Patients are not involved in the conduct, reporting, or dissemination plans of this research.

Recruitment and randomisation

Patients are recruited by the specialised sites via different sources, e.g. by direct referral from either a physician at the Immanuel Hospital Berlin and the outpatient department of the Institute of Social Medicine, Epidemiology and Health Economics at Charité-Universitätsmedizin Berlin, or the Paracelsus-Elena Clinic in Kassel, or by non-personal advertising strategies (e.g. flyers or social media). Participants meeting all the inclusion and no exclusion criteria (Table 1) are assigned to their respective groups (RA, PD, or healthy control) (Figure 1) for the cross-sectional study after written informed consent. Half of the patients from the RA group and half of the patients from the PD group is selected to take part in the longitudinal part of the study, including the fasting intervention according to their availability for all 11 visits and their willingness to follow TRE over 12 months. This study is an open-label trial, as blinding is not feasible in fasting interventions.

Table 1: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> Age 18-79 One of the following diagnoses: rheumatoid arthritis (first diagnosis >6 weeks ago), Parkinson's disease OR healthy volunteer Control ("healthy") individuals must be without any evidence of active known or treated RA, without any evidence of active, known or treated central nervous system disease, and without a known family history of idiopathic PD Control individuals should match the RA or PD individuals as closely as possible (sex, age, education) Present written declaration of consent 	<ul style="list-style-type: none"> Gout or proven bacterial arthritis Participation in another study Existing/current eating disorder (bulimia nervosa, anorexia nervosa) within the past 5 years Severe internal disease (e.g. kidney deficiency with creatinine > 2mg/dl) Existing vegan diet or fasting during the last six months Presence or suspicion of atypical PD (e.g. early dementia, early autonomous dysfunction) Diagnosis of chronic inflammatory bowel diseases, celiac disease or colorectal cancer according to the guidelines of the German Society of Gastroenterology Use of anti-psychotic drugs

<ul style="list-style-type: none"> • Ability to understand the patient information and willingness to sign the consent form • Consent to specimen collection and specimen use 	<ul style="list-style-type: none"> • Antibiotic use during the previous 12 months • Start of novel therapy with disease-modifying anti-rheumatic drugs • Pregnancy or breastfeeding women • Contraindication for additional blood draws (e.g. haemoglobin <10) • BMI < 18.5 • Psychiatric illness that limits understanding of the examination protocol (unable to consent)
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269

270 Fasting dietary counselling

271 The fasting group is closely monitored by nutritionists trained in fasting therapy, backed up by
 272 physicians experienced in fasting, from the Charité – Universitätsmedizin Berlin and the Paracelsus-
 273 Elena Clinic to ensure a uniform implementation of the fasting guidelines and the well-being of the
 274 study participants. The monitoring consists of several in person and virtual meetings which held
 275 individually or in group settings. Five meetings including the visits T0 and T2 during the fasting week
 276 as well as a group meeting after PF to ensure a well-managed start to the TRE phase take place. Group
 277 sessions are standardised using a pre-set deck of slides to be discussed during the group meetings
 278 with only minor disease-related differences between the PD and RA groups. All longitudinal
 279 participants receive a study-specific script with information on fasting procedures.

280

281 Medication

282 The medical treatments of the patients are monitored and documented with every clinical visit. The
 283 fasting intervention might necessitate temporary adjustments of several medications e.g. anti-
 284 diabetic and anti-hypertensive drugs as insulin levels and hypertension will be reduced due to lack of
 285 food intake [31].

286

287 Data collection

288 Sample and data collection is performed at the two clinical sites, Charité – Universitätsmedizin Berlin
 289 and Paracelsus-Elena Clinic (Table 2).

290

291 *Table 2: Sampling procedures.*

a) Biochemical samples and procedures

Blood (123 mL at T0, 23 mL at T2-T11)

Stool collection (2 mL at T0 and T3-T11)

Saliva collection (3.5 mL at T0-T11)

Midstream urine (50 mL at T0 -T11)

292

b) Questionnaires

Disease specific

60

PD:

- Disease Activity Score (DAS-28) [48]
- Parkinson's Disease Sleep Scale-2 (PDSS-2) [49]
- Parkinson's Disease Questionnaire-39 (PDQ-39)[50]
- Simplified Disease Index Score (SDAI) [51]
- Funktionsfragebogen Hannover (FFbH-R) [52]
- Movement Disorder Society Unified PD Rating Scale (MDS-UPDRS)[53]
- Non-Motor Symptoms Questionnaire (NMSQ)[54]
- Non-Motor Symptoms Scale (NMSS)[55]

RA:

- Disease Activity Score (DAS-28) [51]
- Non-Motor Symptoms Questionnaire (NMSQ) [54]
- Funktionsfragebogen Hannover (FFbH-R) [52]

Dietary behaviour and lifestyle

- Fasting experience, expectation, and intervention
- Lifestyle
- 24H-Food-recall
- Food Frequency Questionnaire (FFQ)

General health and well-being

- Health Assessment Questionnaire (HAQ)[56]
 - Bristol Stool Scale[57]
 - Quality of Life questionnaire (WHO-5)[58]
 - Hospital Anxiety and Depression Scale (HADS)[59]
-

- Profile of Mood States[60]

293

294

295 **Anthropometric data and questionnaires**

296 The electronic data capture system REDCap[61], a secure web-based application, is used to record all
 297 individual specific data. All data is stored on a secure server infrastructure at the host institution in
 298 Luxembourg. Weight, height, body mass index (BMI), heart rate and blood pressure in sitting and
 299 standing position as well as waist-hip-ratio is determined at every visit. Dietary behaviour,
 300 sociodemographic measurements (age, sex, education level, employment status, marital status),
 301 family history, current and previous illness and co-morbidities, and current medications, as well as
 302 disease-specific data, questionnaires about the well-being of the patients and data on the behavioural
 303 factors are collected at baseline, T6 (week 3), T9 (month 6) and T11 (month 12) (Table 2).
 304 Questionnaires (24h-Food Recall, Bristol Stool Scale) are answered at all visits by the study
 305 participants. Data storage, analysis and exchange are done only in pseudonymised fashion. The
 306 nutritional data is analysed using the Nutrilog 3.20 software (Nutrilog SAS, Marans).

307

308 **Blood samples and parameters**

309 Blood samples are collected at each visit, and immediately used for peripheral blood mononuclear cell
 310 (PBMC) isolation (T0), analysis by the study laboratory and centrifugation to freeze plasma samples at
 311 -80°C (T0-T11). A clinical standard laboratory report is generated after every visit for each study
 312 participant (Table 3). In addition to routine blood parameters, anti-citrullinated protein antibody
 313 (ACPA), zonulin, fatty-acid binding protein 2 (FABP2), and calprotectin levels are measured. Aliquots
 314 are securely stored to account for novel observations and testing of hypotheses.

315

316 *Table 3: Routine blood parameters measured at each timepoint (T0 for cross-sectional study, T0-T11 for longitudinal study)*

Haematology – EDTA- blood	Clinical Chemistry – Serum
Basophils, %	Albumin
Basophils, abs.	ALT, 37°C
Eosinophils, %	Alkaline Phosphatase, 37°C
Eosinophils, abs.	AST, 37°C
Erythrocytes	Bilirubin, total
Haematocrit	Cholinesterase
Haemoglobin	Cholesterol
HbA1c	Creatinine
Leucocytes	hs-CRP
Lymphocytes, %	Glucose, serum
Lymphocytes, abs.	Gamma-GT, 37°C
MCH	HDL-Cholesterol
MCHC	LDL-Cholesterol

MCV	Potassium
Monocytes, %	Sodium
Monocytes, abs.	Total Protein
Neutrophils, %	Triglycerides
Neutrophils, abs.	Uric Acid
Platelets	Urea/BUN
RDW	Proteins – Serum
Reticulocytes	Rheumatoid factor H 35.9
Reticulocytes	Hormones – Serum
Reticulocytes, abs.	Insulin
	TSH (basal)

317

318 Stool, urine and saliva samples

319 The samples listed in Table 2 are collected at each visit, except for stool samples on T2 (fasting week)
 320 and immediately frozen and stored at -80°C. Stool characteristics are recorded at the time of the
 321 sampling. Faecal samples represent the main sample type for resolving the dynamic processes driven
 322 by microbiome in the gut. Also, as the gut microbiome is prone to diurnal fluctuations, the stool
 323 samples are collected in the morning, as far as possible.

324

325 Methods applied to samples

326

327 Biomolecular extractions

328 The collected stool samples undergo a biomolecular extraction procedure to allow isolation of
 329 concomitant DNA, RNA, proteins, peptides and metabolites from single, unique faecal water samples;
 330 this process involves cryo-milling the samples in liquid nitrogen, disassociating metabolites from
 331 membrane and cell wall components in a solvent mixture of methanol, chloroform and water and
 332 lastly proteins and RNA extraction by a methanol/chloroform and phenol buffer [62, 63]. Faecal water
 333 is recovered following centrifugation and filtration, at low-speed or low-flow, respectively, to avoid
 334 cell lysis. Nucleic acids are preserved by the addition of ribonuclease inhibitors and isolated by silica-
 335 column-based techniques. This protocol involves the use of a robotic platform, ensuring a higher level
 336 of standardisation and reproducibility[2].

337

338 Coupled metagenomic and metatranscriptomic analyses

339 Prior to sequencing library preparation, internal standards are introduced to obtain quantitative
 340 sequencing data[64]. Contamination-free metagenomic (MG) and metatranscriptomic (MT) data is
 341 generated, processed and analysed using the Integrated Meta-omics Pipeline (IMP)[45], which
 342 incorporates pre-processing, assembly, gene annotation, mapping of reads, single nucleotide
 343 polymorphism calling, data normalisation as well as analyses of community structure and function in
 344 a fully reproducible software framework based on Docker. The MG and MT data is specifically
 345 screened for enrichments in genes and pathways with known immunogenic properties[65]. The
 346 extracellular biomolecules are linked to specific microbial populations based on the intracellular
 347 metagenomic data [66]. In addition, the sequencing data is mapped against genomes of food
 348 components[44]. The quantitative data is also related to microbial population sizes to determine the

1
2
3 349 contribution of the resolved microbial populations in stool to the extracellular DNA and RNA
4 350 complements[67].
5 351

7 352 **Metaproteomics**

8 353 For the metaproteomic analyses, filtration is used to separate extracellular peptides from the obtained
9 354 (poly)peptides. The resulting smaller fractions are then desalted and analysed without proteolytic
10 355 digestion via liquid chromatography (LC) and mass spectrometry (MS) on an EasyNano-LC coupled
11 356 online to a QExactive-Plus mass spectrometer (ThermoScientific, Waltham, USA). The identification of
12 357 ribosomal peptides is done with an integrated catalogue of MG and MT data, while the non-ribosomal
13 358 peptides are identified using different tools, i.e., MyriMatch, DirecTag as well as CycloBranch[45, 68,
14 359 69]. The metaproteomic data also allows identification of extracellular (poly)peptides with possible
15 360 pathogenic functions including protein misfolding and molecular mimicry[70, 71].
16 361

19 362 **Metabolomics**

20 363 Metabolomic data is analysed using a combination of targeted and untargeted approaches [44, 63,
21 364 72]. This highlights the major metabolite classes produced by the gut microbiome with an effect on
22 365 human physiology including organic acids, SCFA, lipids, branched-chain fatty acids, branched-chain
23 366 amino acids, vitamins, bile acids and neurotransmitters. Besides external compound calibration series
24 367 for quantification and quality control samples to ensure data normalisation and data acquisition
25 368 quality assessment, the metabolite extraction fluid is fortified with multiple internal standards to
26 369 improve method precision and accuracy[73, 74]. The data is compared to in-house databases and
27 370 public mass spectral libraries to identify known metabolites. The metabolomic data complements the
28 371 metagenomic and metatranscriptomic data and thus allows further establishments of conclusive links
29 372 to metabolic properties in the gut.
30 373

33 374 **Deep immune profiling**

34 375 Deep immune profiling is done using a recently established and optimised panel of metal-labelled
35 376 antibodies together with cytometry coupled to mass spectrometry (MS), the Maxpar Direct Immune
36 377 Profiling System (MDIPA). This approach allows the simultaneous quantification of 38 parameters on
37 378 single cells. Whole blood is stained with the MDIPA kit and stabilised with Proteomic stabiliser Prot-1
38 379 (501351694, Smart Tube Inc., Las Vegas) before storage at -80°C. The quantified immune cells
39 380 included in the MDIPA panel are CD3+, CD4+, CD8+, monocytes, dendritic cells, granulocytes, MAIT, T
40 381 cells, NK and B cells[75]. Cytokine expression profiles is analysed on blood plasma using the Human
41 382 Luminex performance Cytokine Panel (R&D Systems Europe, Abingdon), measuring CCL3, CCL4, CCL5,
42 383 GM-CSF, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-18, IL-21, IL-27, IL-33, IFN- β ,
43 384 Galectin-1, IFN- γ and TNF- α [65].
44 385

47 386 **Gut-on-a-chip models**

48 387 PBMCs isolated from T0 blood samples are co-cultured with gut-derived microbes under
49 388 physiologically representative conditions using the gut-on-a-chip model HuMiX[76]. This model of the
50 389 human gastrointestinal interface allows the investigation of the interactions between immune,
51 390 epithelial and bacterial cells and specifically the response to fasting in personalised in vitro models.
52 391

56 392 **The Expobiome Map**

57 393 The Expobiome Map (<https://expobiome.lcsb.uni.lu>) illustrates the diverse complex of microbial
58 394 immunogenic molecules, including nucleic acids, (poly)peptides, structural molecules, and
59 395 metabolites. The interactions between this “expobiome” and human immune pathways are encoded

396 in the context of chronic diseases[1]. The ExpoBiome Map is visualised using the MINERVA
397 Platform[77]. Clicking on different elements on the map reveals factors they affect and are affected
398 by, allowing an easier navigation through the complex relationships between individual microbiome
399 components in relation to human disease. The multi-omics data generated in the present study will
400 be integrated with the Map.

401

402 Exploratory analysis of novel host-microbiome interactions

403 Unknown non-ribosomal peptides or metabolite features are associated through correlation with
404 transcripts, proteins and metabolites. Extracellular DNA fragments, as well as transcripts, proteins and
405 ribosomal peptides are linked to their genomic context by using IMP[45]. The data generated by the
406 project will be connected and collated to existing, publicly available datasets.

407

408 Outcome parameters

409 Primary Outcome

410 The primary endpoint of the study is the characterisation of the gut microbiome. The evaluation
411 includes both between-group and within-group differences in the longitudinal study arms with the
412 fasting intervention.

413

414 Secondary Outcome Measures

415 Secondary outcomes include the identification of common and disease-specific molecular signatures
416 and the characterisation of microbiome-derived effector molecules impacting the innate and adaptive
417 immune pathways. Furthermore, several additional parameters mentioned in *Anthropometric data*
418 *and questionnaires* are assessed over a period of 12 months.

419

420 Sample size and power calculation

421 A power calculation using pilot metatranscriptomic data based on faecal extracellular RNA samples
422 was performed to determine the number of subjects to be recruited for the ExpoBiome project. The
423 obtained relative abundances of genera were used for the calculation of the required sample size per
424 group. The power calculation was based on the algorithm as described by Tusher, Tibshirani, and
425 Chu[78]. To achieve a power of 90% (at $\alpha = 0.05$), a total of 50 individuals per group (RA, PD, healthy
426 controls) must be analysed. Considering any possible dropouts, 20% additional subjects are recruited,
427 resulting in a total number of 180 individuals, i.e., 60 per group.

428

429 Adverse events

430 There are no major risks expected for participants. Minor common adverse effects of PF might include
431 headaches, nausea, insomnia, back pain, dyspepsia and fatigue[79]. Any occurring adverse events are
432 recorded at each visit in REDCap[61]. Serious adverse events are communicated to the study
433 coordinator and principal investigator within 24 h of their report.

434

435 Data management, monitoring, analysis and evaluation of data

436 The study participants receive a study ID (pseudonym) which is used for all collected data. Self-
437 administered questionnaires are directly recorded in REDCap. Participant files are kept for at least 10
438 years at the respective clinical sites.

439 Weekly meetings between the study team, the different clinical partners and the principal
440 investigator, ensure a close monitoring of the data. Any occurring adverse events or other issues are
441 thus handled immediately.

1
2
3 442 Different statistical tests are performed according to the nature of the data. A premature termination
4 443 of the study is not envisaged; therefore, no interim analysis is done. Different correlation measures
5 444 are applied, including Spearman correlation, mutual information on discretised data, distance
6 445 correlation, maximum information criterion, local similarity analysis and the bioenv approach.
7 446 Comparison across all omic levels allows identification of common and disease-specific signatures.
8 447 Multivariate machine learning is used to link different data features to observed patterns.
9 448 The longitudinal part of the study continues for a period of 12 months. After finalisation of this period,
10 449 there is no follow-up of the participants. Interesting findings will be further validated using the existing
11 450 sample set and analyses may be performed on additionally collected samples.
12 451 The SPIRIT - checklist (Standard Protocol Items: Recommendations for Interventional Trials) was used
13 452 to write this protocol [80].
14
15
16

17 453 **Trial status**

18
19 454 The recruitment for the ExpoBiome study started in April 2021 and is currently ongoing. All study
20 455 participants should be recruited by the end of 2022. The sample collection will take place from April
21 456 2021 to November 2023.
22 457
23
24

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28 461
29
30

31 462 **Author contributions:**

32 463 Study design and protocol were done by Bérénice Hansen, Cédric C. Laczny, Jochen G. Schneider,
33 464 Paul Wilmes; the interventional concept was drawn by Etienne Hanslian, Daniela Liebscher, Andreas
34 465 Michalsen, Anika Hartmann, Brit Mollenhauer, Sebastian Schade, Nico Steckhan, Jochen G.
35 466 Schneider, Paul Wilmes; the clinical trial was designed and is conducted by Etienne Hanslian, Daniela
36 467 Liebscher, Andreas Michalsen, Anika Hartmann, Brit Mollenhauer, Sebastian Schade; the procured
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39 470 Schneider, Paul Wilmes, Kirsten Roomp; the initial draft of the manuscript and coordination of the
40 471 editing process were performed by Bérénice Hansen; the protocol preparation has been done by
41 472 Bérénice Hansen, Audrey Frachet-Bour, Janine Habier; the planning of the data analysis was done by
42 473 Cédric C. Laczny, Jochen G. Schneider, Paul Wilmes, Kirsten Roomp, Velma T.E. Aho, Marek
43 474 Ostaszewski; all authors contributed equally with edits, comments and feedback, read and approved
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53
54

55 481 **Competing interests statement**

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Supplements

The SPIRIT checklist was used to write our report[80].

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683 Figure Legends

684 Figure 1. Study design. This figure illustrates the study design with five different arms in
685 total, two of which continue with the longitudinal part of the study. Visits take place at the
686 clinical sites at each timepoint and include the collection of the displayed samples. This
687 image was generated using Biorender software (<http://www.biorender.com>). T, timepoint;
688 W, week; D, day; M, month.

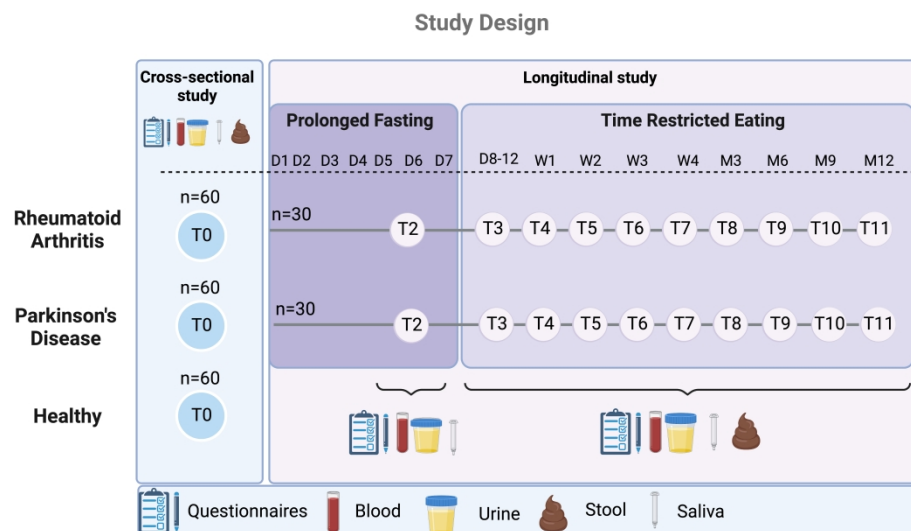


Figure 1. Study design. This figure illustrates the study design with five different arms in total, two of which continue with the longitudinal part of the study. Visits take place at the clinical sites at each timepoint and include the collection of the displayed samples. This image was generated using Biorender software (<http://www.biorender.com>). T, timepoint; W, week; D, day; M, month.

279x177mm (600 x 600 DPI)

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRIT reporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Gøtzsche PC, Altman DG, Mann H, Berlin J, Dickersin K, Hróbjartsson A, Schulz KF, Parulekar WR, Krleža-Jerić K, Laupacis A, Moher D. SPIRIT 2013 Explanation and Elaboration: Guidance for protocols of clinical trials. *BMJ*. 2013;346:e7586

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	n/a
Protocol version	#3	Date and version identifier	n/a
Funding	#4	Sources and types of financial, material, and other support	12
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
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7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	1, 12
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
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16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	1, 12
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
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24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	5
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
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32	Background and	#6b	Explanation for choice of comparators	5
33	rationale: choice of			
34	comparators			
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37	Objectives	#7	Specific objectives or hypotheses	5
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	5
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
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46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
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53	Study setting	#9	Description of study settings (eg, community clinic, academic	6
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
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1	Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
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6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	5,7
7	description			
8				
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10	Interventions:	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	5,7
11	modifications			
12				
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15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	7
16	adherence			
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21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	6
22	concomitant care			
23				
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25	Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	11
26				
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34	Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	5
35				
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40	Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	11
41				
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45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	5
46				
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48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
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54	Allocation: sequence	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be	n/a
55	generation			
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provided in a separate document that is unavailable to those who enrol participants or assign interventions

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4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),
6			describing any steps to conceal the sequence until interventions
7	mechanism		are assigned
8			
9			
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11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol
12	implementation		participants, and who will assign participants to interventions
13			
14			
15	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial
16			participants, care providers, outcome assessors, data analysts),
17			and how
18			
19			
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,
21	emergency unblinding		and procedure for revealing a participant's allocated intervention
22			during the trial
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24			
25	Methods: Data		
26	collection,		
27	management, and		
28	analysis		
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32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and
33			other trial data, including any related processes to promote data
34			quality (eg, duplicate measurements, training of assessors) and a
35			description of study instruments (eg, questionnaires, laboratory
36			tests) along with their reliability and validity, if known.
37			Reference to where data collection forms can be found, if not in
38			the protocol
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43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,
44	retention		including list of any outcome data to be collected for participants
45			who discontinue or deviate from intervention protocols
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49	Data management	#19	Plans for data entry, coding, security, and storage, including any
50			related processes to promote data quality (eg, double data entry;
51			range checks for data values). Reference to where details of data
52			management procedures can be found, if not in the protocol
53			
54			
55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary
56			outcomes. Reference to where other details of the statistical
57			analysis plan can be found, if not in the protocol
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1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	12
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	12
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
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10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	12
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
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22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	n/a
23	interim analysis		including who will have access to these interim results and make	
24			the final decision to terminate the trial	
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27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	12
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
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33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	n/a
34			whether the process will be independent from investigators and	
35			the sponsor	
36				
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38	Ethics and			
39	dissemination			
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41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	2
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	2
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
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53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	6
54			participants or authorised surrogates, and how (see Item 32)	
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1	Consent or assent:	#26b	Additional consent provisions for collection and use of	6
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
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6	Confidentiality	#27	How personal information about potential and enrolled	12
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
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11	Declaration of interests	#28	Financial and other competing interests for principal investigators	14
12			for the overall trial and each study site	
13				
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15	Data access	#29	Statement of who will have access to the final trial dataset, and	12
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
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20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	n/a
21	care		compensation to those who suffer harm from trial participation	
22				
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24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	2
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
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33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	14
34	authorship		professional writers	
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37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
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41	Appendices			
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43	Informed consent	#32	Model consent form and other related documentation given to	6
44	materials		participants and authorised surrogates	
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47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	7
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
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 54 <https://www.goodreports.org/>, a tool made by the [EQUATOR Network](#) in collaboration with [Penelope.ai](#)

BMJ Open

Protocol for a multicentre cross-sectional, longitudinal ambulatory clinical trial in rheumatoid arthritis and Parkinson's disease patients analysing the relation between the gut microbiome, fasting and immune status (ExpoBiome)

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2022-071380.R2
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4 1 **Protocol for a multicentre cross-sectional, longitudinal ambulatory**
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6 2 **clinical trial in rheumatoid arthritis and Parkinson's disease patients**
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8 3 **analysing the relation between the gut microbiome, fasting and**
9
10 4 **immune status (ExpoBiome)**

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Abstract

Introduction

Chronic inflammatory diseases like rheumatoid arthritis (RA) and neurodegenerative disorders like Parkinson's disease (PD) have recently been associated with a decreased diversity in the gut microbiome, emerging as key driver of various diseases. The specific interactions between gut-borne microorganisms and host pathophysiology remain largely unclear. The microbiome can be modulated by interventions comprising nutrition.

The aim of our clinical study is to (1) examine effects of prolonged fasting and time-restricted eating (TRE) on the outcome parameters and the immunophenotypes of RA and PD with (2) special consideration of microbial taxa and molecules associated with changes expected in (1), and (3) identify factors impacting the disease course and treatment by in-depth screening of microorganisms and molecules in personalised HuMiX gut-on-chip models, to identify novel targets for anti-inflammatory therapy.

Methods and Analysis

This trial is an open-label, multicentre, controlled clinical trial consisting of a cross-sectional and a longitudinal study. A total of 180 patients is recruited. For the cross-sectional study, 60 patients with PD, 60 patients with RA and 60 healthy controls are recruited at two different, specialized clinical sites. For the longitudinal part, 30 patients with PD and 30 patients with RA undergo 5-7 days of prolonged fasting (PF) followed by TRE (16:8) for a period of 12 months. One baseline visit takes place before the PF intervention and 10 follow-up visits will follow over a period of 12 months (April 2021 to November 2023).

Ethics and dissemination

Ethical approval was obtained to plan and conduct the trial from the institutional review board of the Charité-Universitätsmedizin Berlin (EA1/204/19), the ethics committee of the state medical association (Landesärztekammer) of Hessen (2021-2230-zvBO) and the Ethics Review Panel (ERP) of the University of Luxembourg (ERP 21-001-A ExpoBiome). The results of this study will be disseminated through peer-reviewed publications, scientific presentations and social media.

Trial registration number at clinicaltrials.gov:

NCT04847011

Key words: Microbiome, fasting therapy, intermittent fasting, time restricted eating, chronic disease, rheumatoid arthritis, Parkinson's disease, nutrition, chronic diseases, ExpoBiome, inflammation, gut on a chip, HuMiX, immunophenotype, metagenomics, metatranscriptomics, metaproteomics, metabolomics

Strengths and limitations of the study

- The participants of the longitudinal study will be closely monitored for 12 months and routine blood parameters as well as anthropometric data and questionnaires will be precisely documented.

- 94 • This study will identify novel microbiome-derived common and disease-associated
95 molecules involved in immune system modulation in two major chronic diseases: RA
96 and PD.
- 97 • This study aims at also identifying novel targeted pathways to control chronic
98 inflammatory conditions in the future.
- 99 • A limitation is the heterogeneity of the cohorts regarding age and sex, which is due to
100 the prevalence of the diseases: RA is more common in women, while PD is more
101 common in men and has a later disease onset.
- 102 • A bias exists in choosing RA and PD as chronic disorders to study immunophenotypes
103 although generalisable results are targeted.
104

105 Introduction (1339)

106
107 The human microbiome is emerging as a key driver of various diseases through its complex of distinct
108 yet connected biomolecules (referred to as the “*expobiome*”)[1, 2]. The expobiome comprises a
109 diverse set of nucleic acids, polypeptides and metabolites which, in the gut alone, are present in
110 substantial concentrations[1]. However, the specific interactions between gut-borne microorganisms
111 and host (patho)physiology remain largely unknown. Although host genetics shape the composition
112 of the gut microbiome, the latter is particularly influenced by non-genetic factors such as lifestyle and
113 diet[3, 4]. Therefore, the microbiome is a plausible target to modify health outcomes.

114
115 Individuals suffering from chronic diseases, including autoimmune, metabolic, and neurodegenerative
116 diseases as well as cancer, often present alterations in their gut microbiome composition. These shifts
117 are typically characterised by an overgrowth of one or several microbial species with likely adverse
118 effects as well as a decrease in beneficial taxa[5]. Such imbalances are referred to as dysbiosis.
119 Although structural microbiome changes are clearly detectable, the mechanistic or functional
120 consequences of dysbiosis are still largely unknown. However, they may result in dysregulated
121 interactions with the immune system[6]. Considering the intricacy of the immune system, the
122 question arises whether the observed microbiome changes are cause or consequence of disease. This
123 implies that, in addition to the genetic predisposition of the host, the gut microbiome needs to be
124 considered a potential pathogenic factor or major driver of disease onset and course[3, 4].
125

126 RA and PD are two specific examples representing dysregulated microbiome-immune system
127 interactions [7, 8]. RA is a multifactorial, chronic, and systemic autoimmune disease, primarily
128 affecting the lining of the synovial joints with a higher risk and younger age for disease onset in women
129 and a global prevalence of 1%[9, 10]. The exact disease pathogenesis is still unclear and no cure for
130 RA currently exists. In addition to the common local articular symptoms of RA, systemic comorbidities
131 can affect the vasculature, metabolism and bones[11]. Besides various environmental risk factors e.g.
132 smoking and a Western diet, the host microbiome is associated with the pathophysiology of the
133 disease[12]. The diversity of the gut microbiome has been reported to be decreased in individuals with
134 RA, compared with the general population, and is correlated with disease duration, activity, and
135 autoantibody levels[13, 14]. Studies in murine models also report that autoimmune arthritis is strongly
136 attenuated under germ-free conditions[15]. The introduction of specific bacteria, e.g. segmented
137 filamentous bacteria, into germ-free animals or oral infection with *Porphyromonas gingivalis* drive
138 autoimmune arthritis through activation of T helper cells[15]. Several different taxa, including
139 *Prevotella copri*, *Lactobacillus* spp. and *Colinsella* spp. are enriched in the gut microbiome of patients
140 with RA and correlate positively with disease markers e.g. immunoglobulins IgA and IgG , while other
141 taxa like *Haemophilus* spp. and *Faecalibacterium* spp. are typically found at lower abundances in

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3 142 patients with RA compared to healthy individuals[13, 16, 17]. Alterations of the gut microbiome may
4 143 therefore have an important impact on RA pathophysiology[12].
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6 145 PD affects 0.4-2% of the population over 65 years worldwide and is the second most common
7 146 progressive neurodegenerative disease with men being 1.5 times more likely to be affected than
8 147 women[18]. Cardinal symptoms are motor deficiencies such as tremor and rigidity, but also include a
9 148 wide range of non-motor symptoms, such as hyposmia, depression, insomnia or cognitive impairment,
10 149 severely impacting patients' quality of life[19]. Aggregations of the protein α -synuclein in the
11 150 dopaminergic substantia nigra represent the main neuropathological manifestations[20]. PD-
12 151 associated loss of dopaminergic neurons involves mechanisms of inflammatory and autoimmune
13 152 responses with microglial activity as a major driver [21].Dysbiosis of the gut microbiome has been
14 153 associated with the characteristic motor deficits and pathophysiological changes in the enteric and
15 154 central nervous systems in animal studies. Increased relative abundances of the genera *Akkermansia*,
16 155 *Bifidobacterium*, *Lactobacillus*, and *Methanobrevibacter* and decreased abundances in
17 156 *Faecalibacterium* and *Roseburia* have been reported[22, 23]. Two recently published clinical-trials
18 157 with prebiotic supplementation in PD observed a shift in gut microbiome composition, an increase in
19 158 short-chain fatty acids (SCFA) and a reduction in non-motor-symptoms [24, 25]. Most patients with
20 159 PD suffer from gastrointestinal symptoms such as constipation and irritable bowel syndrome (IBS) -
21 160 like symptoms[26]. The gut-brain axis, e.g. by-products produced by the gut microbiome, may
22 161 contribute to the production of α -synuclein aggregates in the enteric nervous system[27]. In addition,
23 162 increased intestinal permeability[28] as driver for enteric inflammation occur in PD and substantiate
24 163 a role of peripheral inflammation in the initiation and the progression of the disease[29].
25 164

26 165 One factor with known major impact on the gut microbiome and on chronic diseases is diet[7]. Dietary
27 166 approaches as fasting have already been used by Hippocrates in the 5th century BCE and have been
28 167 applied ever since by numerous medical schools to treat acute and chronic diseases [30-32]. Various
29 168 practices of caloric restriction through fasting have repeatedly shown remarkable health benefits[33,
30 169 34]. Maifeld *et al.* found that a 5-day fast followed by a modified Dietary Approach to Stop
31 170 Hypertension (DASH), with additional emphasis on plant-based and Mediterranean diets, reduced
32 171 systolic blood pressure, BMI, and the need for antihypertensive medications at three months post
33 172 intervention compared with DASH alone [35].
34 173

35 174 Furthermore, Choi *et al.* demonstrated that cycles of a fasting-mimicking diet suppress autoimmunity
36 175 and stimulate remyelination via oligodendrocyte regeneration in a murine experimental autoimmune
37 176 encephalomyelitis (EAE) model[36]. Jordan *et al.* described a reduction in monocyte metabolic and
38 177 inflammatory activity after a short-term fast and conclude that fasting attenuates chronic
39 178 inflammatory diseases without compromising monocyte capacity for mobilisation during acute
40 179 infectious inflammation and tissue repair[37].
41 180

42 181 These improvements can, however, typically only be maintained for a limited period of time, and the
43 182 symptoms can reappear after reintroduction of the patients' standard diet. Hence, protocols to sustain
44 183 these beneficial effects are of utmost importance. In mouse models of PD, intermittent fasting (IF) has
45 184 led to several improvements including decreased excitotoxicity, reduced neurodegeneration and
46 185 protection against autonomic dysfunction, motor and cognitive decline[38].
47 186

48 187 IF and PF may have potent immunomodulatory effects which may partially be mediated by the gut
49 188 microbiome and the fasting induced alterations of the latter[39]. These microbial shifts include
50 189 upregulation of *Akkermansia muciniphila*, *Bacteroides fragilis*, other *Bacteroides* spp., Proteobacteria,
51 190 and butyric acid producing *Lachnospiraceae*, but also *Odoribacter*, which is negatively associated with
52 191 blood pressure[35, 40]. Interestingly, an overall decrease of the Firmicutes/Bacteroidetes ratio could
53 192 be observed, a high ratio is commonly associated with several pathologies, including RA [41].
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194 A potential mechanism underlying the observed beneficial effects induced by dietary interventions
195 might be a direct gut microbiome-immune system interaction by pattern recognition. The microbiome
196 can regulate the intestinal innate immune system by modulating toll-like receptor (TLR) expression on
197 immunosensor cell surface through microbe-associated molecular patterns (MAMPs), which can
198 consequently trigger cytokine production and up-regulation of molecules on antigen presenting cells,
199 leading to activation of T cells[42]. Therefore, a change in gut microbiome composition can lead to
200 different outcomes in immune signalling pathways and either favour or suppress inflammation and
201 autoimmunity.

202
203 The impact and importance of the gut microbiome on human physiology and its potential
204 modifications by nutrition and dietary patterns, have been underestimated for centuries[43]. Reasons
205 may include missing standardised therapeutic protocols, the interindividual variability in the response
206 to fasting, lack of knowledge about possible adverse effects, and difficulties in the interpretation of
207 underlying mechanisms seen in clinical trials, but also in the comparably low potential for achieving
208 economic revenue or scientific impact[8].

209
210 Modern experimental approaches and computational integration allow a multi-layer analysis of
211 digestive processes in low caloric settings including the gut microbiome[44]. These technological
212 developments also permit a closer investigation of the link between the immune system and severe
213 caloric restriction.

214
215 To our knowledge no clinical trials have been investigating the connection between IF or PF and PD in
216 humans so far[38]. Our study aims to elucidate the causal relationship between the gut microbiome
217 and the immune system. To do so, we will use analyses of the molecular basis of human-microbiome
218 interactions enabled by high throughput methodologies such as the combination of metagenomics,
219 metatranscriptomics and metaproteomics. Moreover, we are aiming at identifying new genes,
220 proteins, metabolites, and host pathways facilitating the development of novel diagnostic and
221 therapeutic tools[45, 46].

222

223 **Methods and Analysis (3122)**

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225 **Study objectives**

226 The first objective of the study is to define specific gut microbiome-derived molecules in RA and PD,
227 compared to healthy individuals, and relate this information to the immunophenotypes of the
228 individuals. The second objective is to identify and track common and disease-specific molecular
229 signatures to predict the outcome of a gut microbiome-targeted therapeutic intervention, here
230 fasting, on inflammation-driven symptoms in RA and PD. The third objective of the study is to identify
231 and validate microbiome-derived effector molecules which downregulate pro-inflammatory innate
232 and adaptive immune pathways.

233

234 **Study design**

235 The ExpoBiome cohort consists of 180 adult individuals, meeting the exclusion and inclusion criteria
236 (Table 1), for the cross-sectional study (objectives 1 and 3) and 60 adult individuals for the longitudinal
237 study (objectives 2 and 3). There are five different arms in total: (1) RA – cross-sectional arm [60
238 patients], (2) PD – cross-sectional arm [60 patients], and (3) healthy controls – cross-sectional arm [60
239 patients], (4) RA – longitudinal arm [30 patients], (5) PD – longitudinal arm [30 patients] (Figure1).

240

At the first visit (T0), patients answer several questionnaires, and blood, urine, saliva, and stool samples are obtained (Table 2). The longitudinal arms (4) and (5) undergo a 5–7-day PF with a dietary energy supply of max. 350-400 kcal per day with vegetable or grain broths as well as fresh vegetable juices[31, 40]. After the PF, the longitudinal arms follow a dietary regimen including the concept of TRE for a period of 12 months following the 16:8 pattern[47]. This means that food intake is allowed ad libitum for 8 h, followed by 16 h of fasting where no food should be consumed. The intake of non-caloric beverages, e.g., water, unsweetened tea or coffee is, however, allowed. The participants attend one follow-up visit (T2) during the PF and 9 follow-up visits during the 12 months of TRE (Figure 1).

Patient and Public Involvement

Feedback of patients during former clinical trials at the study centre in Berlin was integrated in the planning and design of the fasting intervention of this study. Patients are not involved in the conduct, reporting, or dissemination plans of this research.

Recruitment and randomisation

Patients are recruited by the specialised sites via different sources, e.g., by direct referral from either a physician at the Immanuel Hospital Berlin and the outpatient department of the Institute of Social Medicine, Epidemiology and Health Economics at Charité-Universitätsmedizin Berlin, or the Paracelsus-Elena Clinic in Kassel, or by non-personal advertising strategies (e.g. flyers or social media). For PD, the patients are screened by an experienced movement disorders specialist for featuring at least two of resting tremor, bradykinesia, and rigidity according to the United Kingdom Parkinson's Disease Society Brain Bank criteria[48]. Additionally, patients must show evidence of a dopaminergic deficit, either with DaTScan imaging or with a clear response to dopaminergic drugs. Motor and non-motor symptoms are assessed with the MDS-UPDRS (part I – IV) including the Hoehn and Yahr (severity) scale[49]. Additional PD-specific scales as Parkinson's Disease Sleep Scale-2, Parkinson's Disease Questionnaire-39, Non-Motor Symptoms Questionnaire and Non-Motor Symptoms Scale are used.

For patients with RA, the diagnosis has been made prior to the study by an experienced rheumatologist according to the European League Against Rheumatism (EULAR) criteria[50]. All clinical stages of RA will be included. We excluded patients with a BMI <18.5, as this indicates underweight, and fasting is not recommended. We did, however, not include an upper limit as fasting might be especially beneficial for patients with a BMI >24.9 and more than 60% of patients with RA are classified as overweight or obese[51]. For comorbidities we excluded mainly diseases which are known to interfere with the gut microbiome and might be potential confounders.

The chosen exclusion criteria will optimize the pairing process of healthy controls and patients with either RA or PD. However, as we have two diseases with different anthropometric characteristics (including age, gender, BMI) and only one control group, adding additional inclusion and exclusion criteria in the recruitment process would compromise on optimized matching. Furthermore, for the longitudinal part of the study, each patient will serve as his/her own control over time. Participants meeting all the inclusion and no exclusion criteria (Table 1) are assigned to their respective groups (RA, PD, or healthy control) (Figure 1) for the cross-sectional study after written informed consent. Half of the patients from the RA group and half of the patients from the PD group is selected to take part in the longitudinal part of the study, including the fasting intervention according to their availability for all 11 visits and their willingness to follow TRE over 12 months. This study is an open-label trial, as blinding is not feasible in fasting interventions.

Table 1: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
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<ul style="list-style-type: none"> • Age 18-79 • One of the following diagnoses: rheumatoid arthritis (first diagnosis >6 weeks ago), Parkinson's disease OR healthy volunteer • Control ("healthy") individuals must be without any evidence of active known or treated RA, without any evidence of active, known or treated central nervous system disease, and without a known family history of idiopathic PD • Control individuals should match the RA or PD individuals as closely as possible (sex, age, education) • Present written declaration of consent • Ability to understand the patient information and willingness to sign the consent form • Consent to specimen collection and specimen use 	<ul style="list-style-type: none"> • Gout or proven bacterial arthritis • Participation in another study • Existing/current eating disorder (bulimia nervosa, anorexia nervosa) within the past 5 years • Severe internal disease (e.g. kidney deficiency with creatinine > 2mg/dl) • Existing vegan diet or fasting during the last six months • Presence or suspicion of atypical PD (e.g. early dementia, early autonomous dysfunction) • Diagnosis of chronic inflammatory bowel diseases, celiac disease or colorectal cancer according to the guidelines of the German Society of Gastroenterology • Use of anti-psychotic drugs • Antibiotic use during the previous 12 months • Start of novel therapy with disease-modifying anti-rheumatic drugs • Pregnancy or breastfeeding women • Contraindication for additional blood draws (e.g. haemoglobin <10) • BMI < 18.5 • Psychiatric illness that limits understanding of the examination protocol (unable to consent)
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290 Fasting dietary counselling

291 The fasting group is closely monitored by nutritionists trained in fasting therapy, backed up by
 292 physicians experienced in fasting, from the Charité – Universitätsmedizin Berlin and the Paracelsus-
 293 Elena Clinic to ensure a uniform implementation of the fasting guidelines and the well-being of the
 294 study participants. The monitoring consists of several in person and virtual meetings which held
 295 individually or in group settings. Five meetings including the visits T0 and T2 during the fasting week
 296 as well as a group meeting after PF to ensure a well-managed start to the TRE phase take place. Group
 297 sessions are standardised using a pre-set deck of slides to be discussed during the group meetings
 298 with only minor disease-related differences between the PD and RA groups. All longitudinal
 299 participants receive a study-specific script with information on fasting procedures. Although the
 300 adherence of the patients cannot be profoundly controlled in the ambulatory setting, the blood
 301 samples will allow us to have additional insight into the nutritional habits as well as the fasting state
 302 of the patients on the day of the visit (blood glucose levels).

303

304 Medication

305 The medical treatments of the patients are monitored and documented with every clinical visit. The
 306 fasting intervention might necessitate temporary adjustments of several medications e.g., anti-
 307 diabetic and anti-hypertensive drugs as insulin levels and hypertension will be reduced due to lack of
 308 food intake [31].
 309

310 Data collection

311 Sample and data collection is performed at the two clinical sites, Charité – Universitätsmedizin Berlin
 312 and Paracelsus-Elena Clinic (Table 2).
 313

314 *Table 2: Sampling procedures.*

a) Biochemical samples and procedures

Blood (123 mL at T0, 23 mL at T2-T11)

Stool collection (2 mL at T0 and T3-T11)

Saliva collection (3.5 mL at T0-T11)

Midstream urine (50 mL at T0 -T11)

315

b) Questionnaires

Disease specific

PD:

- Disease Activity Score (DAS-28) [52]
- Parkinson's Disease Sleep Scale-2 (PDSS-2) [53]
- Parkinson's Disease Questionnaire-39 (PDQ-39)[54]
- Simplified Disease Index Score (SDAI) [55]
- Funktionsfragebogen Hannover (FFbH-R) [56]
- Movement Disorder Society Unified PD Rating Scale (MDS-UPDRS)[57]
- Non-Motor Symptoms Questionnaire (NMSQ)[58]
- Non-Motor Symptoms Scale (NMSS)[59]

RA:

- Disease Activity Score (DAS-28) [55]

-
- Non-Motor Symptoms Questionnaire (NMSQ) [58]
 - Funktionsfragebogen Hannover (FFbH-R) [56]

Dietary behaviour and lifestyle

- Fasting experience, expectation, and intervention
- Lifestyle
- 24H-Food-recall
- Food Frequency Questionnaire (FFQ)

General health and well-being

- Health Assessment Questionnaire (HAQ)[60]
 - Bristol Stool Scale[61]
 - Quality of Life questionnaire (WHO-5)[62]
 - Hospital Anxiety and Depression Scale (HADS)[63]
 - Profile of Mood States[64]
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318 Anthropometric data and questionnaires

319 The electronic data capture system REDCap[65], a secure web-based application, is used to record all
320 individual specific data. All data is stored on a secure server infrastructure at the host institution in
321 Luxembourg. Weight, height, body mass index (BMI), heart rate and blood pressure in sitting and
322 standing position as well as waist-hip-ratio is determined at every visit. Dietary behaviour,
323 sociodemographic measurements (age, sex, education level, employment status, marital status),
324 family history, current and previous illness and co-morbidities, and current medications, as well as
325 disease-specific data, questionnaires about the well-being of the patients and data on the behavioural
326 factors are collected at baseline, T6 (week 3), T9 (month 6) and T11 (month 12) (Table 2).
327 Questionnaires (24h-Food Recall, Bristol Stool Scale) are answered at all visits by the study
328 participants. Data storage, analysis and exchange are done only in pseudonymised fashion. The
329 nutritional data is analysed using the Nutrilog 3.20 software (Nutrilog SAS, Marans).

330

331 Blood samples and parameters

332 Blood samples are collected at each visit, and immediately used for peripheral blood mononuclear cell
333 (PBMC) isolation (T0), analysis by the study laboratory and centrifugation to freeze plasma samples at
334 -80°C (T0-T11). A clinical standard laboratory report is generated after every visit for each study
335 participant (Table 3). In addition to routine blood parameters, anti-citrullinated protein antibody

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336 (ACPA), zonulin, fatty-acid binding protein 2 (FABP2), and calprotectin levels are measured. Aliquots
 337 are securely stored to account for novel observations and testing of hypotheses.

338

339 *Table 3: Routine blood parameters measured at each timepoint (T0 for cross-sectional study, T0-T11 for longitudinal study)*

Haematology – EDTA- blood	Clinical Chemistry – Serum
Basophils, %	Albumin
Basophils, abs.	ALT, 37°C
Eosinophils, %	Alkaline Phosphatase, 37°C
Eosinophils, abs.	AST, 37°C
Erythrocytes	Bilirubin, total
Haematocrit	Cholinesterase
Haemoglobin	Cholesterol
HbA1c	Creatinine
Leucocytes	hs-CRP
Lymphocytes, %	Glucose, serum
Lymphocytes, abs.	Gamma-GT, 37°C
MCH	HDL-Cholesterol
MCHC	LDL-Cholesterol
MCV	Potassium
Monocytes, %	Sodium
Monocytes, abs.	Total Protein
Neutrophils, %	Triglycerides
Neutrophils, abs.	Uric Acid
Platelets	Urea/BUN
RDW	Proteins – Serum
Reticulocytes	Rheumatoid factor H 35.9
Reticulocytes	Hormones – Serum
Reticulocytes, abs.	Insulin
	TSH (basal)

340

341 Stool, urine and saliva samples

342 The samples listed in Table 2 are collected at each visit, except for stool samples on T2 (fasting week)
 343 and immediately frozen and stored at -80°C. Stool characteristics are recorded at the time of the
 344 sampling. Faecal samples represent the main sample type for resolving the dynamic processes driven
 345 by microbiome in the gut. Also, as the gut microbiome is prone to diurnal fluctuations, the stool
 346 samples are collected in the morning, as far as possible.

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4 348 **Methods applied to samples**

5 349

6 349 7 8 350 **Biomolecular extractions**

9 351 The collected stool samples undergo a biomolecular extraction procedure to allow isolation of
10 352 concomitant DNA, RNA, proteins, peptides and metabolites from single, unique faecal water samples;
11 353 this process involves cryo-milling the samples in liquid nitrogen, disassociating metabolites from
12 354 membrane and cell wall components in a solvent mixture of methanol, chloroform and water and
13 355 lastly proteins and RNA extraction by a methanol/chloroform and phenol buffer [66, 67]. Faecal water
14 356 is recovered following centrifugation and filtration, at low-speed or low-flow, respectively, to avoid
15 357 cell lysis. Nucleic acids are preserved by the addition of ribonuclease inhibitors and isolated by silica-
16 358 column-based techniques. This protocol involves the use of a robotic platform, ensuring a higher level
17 359 of standardisation and reproducibility[2].
18 359
19 360

20 360 21 361 **Coupled metagenomic and metatranscriptomic analyses**

22 362 Prior to sequencing library preparation, internal standards are introduced to obtain quantitative
23 363 sequencing data[68]. Contamination-free metagenomic (MG) and metatranscriptomic (MT) data is
24 364 generated, processed and analysed using the Integrated Meta-omics Pipeline (IMP)[45], which
25 365 incorporates pre-processing, assembly, gene annotation, mapping of reads, single nucleotide
26 366 polymorphism calling, data normalisation as well as analyses of community structure and function in
27 367 a fully reproducible software framework based on Docker. The MG and MT data is specifically
28 368 screened for enrichments in genes and pathways with known immunogenic properties[69]. The
29 369 extracellular biomolecules are linked to specific microbial populations based on the intracellular
30 370 metagenomic data [70]. In addition, the sequencing data is mapped against genomes of food
31 371 components[44]. The quantitative data is also related to microbial population sizes to determine the
32 372 contribution of the resolved microbial populations in stool to the extracellular DNA and RNA
33 373 complements[71].
34 373
35 374

36 374 37 375 **Metaproteomics**

38 375
39 376 For the metaproteomic analyses, filtration is used to separate extracellular peptides from the obtained
40 377 (poly)peptides. The resulting smaller fractions are then desalted and analysed without proteolytic
41 378 digestion via liquid chromatography (LC) and mass spectrometry (MS) on an EasyNano-LC coupled
42 379 online to a QExactive-Plus mass spectrometer (ThermoScientific, Waltham, USA). The identification of
43 380 ribosomal peptides is done with an integrated catalogue of MG and MT data, while the non-ribosomal
44 381 peptides are identified using different tools, i.e., MyriMatch, DirecTag as well as CycloBranch[45, 72,
45 382 73]. The metaproteomic data also allows identification of extracellular (poly)peptides with possible
46 383 pathogenic functions including protein misfolding and molecular mimicry[74, 75].
47 383
48 384

49 384 50 385 **Metabolomics**

51 386 Metabolomic data is analysed using a combination of targeted and untargeted approaches [44, 67,
52 387 76]. This highlights the major metabolite classes produced by the gut microbiome with an effect on
53 388 human physiology including organic acids, SCFA, lipids, branched-chain fatty acids, branched-chain
54 389 amino acids, vitamins, bile acids and neurotransmitters. Besides external compound calibration series
55 390 for quantification and quality control samples to ensure data normalisation and data acquisition
56 391 quality assessment, the metabolite extraction fluid is fortified with multiple internal standards to
57 392 improve method precision and accuracy[77, 78]. The data is compared to in-house databases and
58 393 public mass spectral libraries to identify known metabolites. The metabolomic data complements the
59 393
60

394 metagenomic and metatranscriptomic data and thus allows further establishments of conclusive links
395 to metabolic properties in the gut.

396

397 Deep immune profiling

398 Deep immune profiling is done using a recently established and optimised panel of metal-labelled
399 antibodies together with cytometry coupled to mass spectrometry (MS), the Maxpar Direct Immune
400 Profiling System (MDIPA). This approach allows the simultaneous quantification of 38 parameters on
401 single cells. Whole blood is stained with the MDIPA kit and stabilised with Proteomic stabiliser Prot-1
402 (501351694, Smart Tube Inc., Las Vegas) before storage at -80°C. The quantified immune cells
403 included in the MDIPA panel are CD3+, CD4+, CD8+, monocytes, dendritic cells, granulocytes, MAIT, T
404 cells, NK and B cells[79]. Cytokine expression profiles is analysed on blood plasma using the Human
405 Luminex performance Cytokine Panel (R&D Systems Europe, Abingdon), measuring CCL3, CCL4, CCL5,
406 GM-CSF, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-18, IL-21, IL-27, IL-33, IFN- β ,
407 Galectin-1, IFN- γ and TNF- α [69].

408

409 Gut-on-a-chip models

410 PBMCs isolated from T0 blood samples are co-cultured with gut-derived microbes under
411 physiologically representative conditions using the gut-on-a-chip model HuMiX[80]. This model of the
412 human gastrointestinal interface allows the investigation of the interactions between immune,
413 epithelial and bacterial cells and specifically the response to fasting in personalised in vitro models.

414

415 The Expobiome Map

416 The Expobiome Map (<https://expobiome.lcsb.uni.lu>) illustrates the diverse complex of microbial
417 immunogenic molecules, including nucleic acids, (poly)peptides, structural molecules, and
418 metabolites. The interactions between this “expobiome” and human immune pathways are encoded
419 in the context of chronic diseases[1]. The ExpoBiome Map is visualised using the MINERVA
420 Platform[81]. Clicking on different elements on the map reveals factors they affect and are affected
421 by, allowing an easier navigation through the complex relationships between individual microbiome
422 components in relation to human disease. The multi-omics data generated in the present study will
423 be integrated with the Map.

424

425 Exploratory analysis of novel host-microbiome interactions

426 Unknown non-ribosomal peptides or metabolite features are associated through correlation with
427 transcripts, proteins, and metabolites. Extracellular DNA fragments, as well as transcripts, proteins
428 and ribosomal peptides are linked to their genomic context by using IMP[45]. The data generated by
429 the project will be connected and collated to existing, publicly available datasets.

430

431 Outcome parameters

432 Primary Outcome

433 The primary endpoint of the study is the characterisation of the gut microbiome. The evaluation
434 includes both between-group and within-group differences in the longitudinal study arms with the
435 fasting intervention.

436

437 Secondary Outcome Measures

438 Secondary outcomes include the identification of common and disease-specific molecular signatures
439 and the characterisation of microbiome-derived effector molecules impacting the innate and adaptive

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3 440 immune pathways. Furthermore, several additional parameters mentioned in *Anthropometric data*
4 441 *and questionnaires* are assessed over a period of 12 months.
5 442

7 443 **Sample size and power calculation**

8 444 A power calculation using pilot metatranscriptomic data based on faecal extracellular RNA samples
9 445 was performed to determine the number of subjects to be recruited for the ExpoBiome project. The
10 446 obtained relative abundances of genera were used for the calculation of the required sample size per
11 447 group. The power calculation was based on the algorithm as described by Tusher, Tibshirani, and
12 448 Chu[82]. To achieve a power of 90% (at $\alpha = 0.05$), a total of 50 individuals per group (RA, PD, healthy
13 449 controls) must be analysed. Considering any possible dropouts, 20% additional subjects are recruited,
14 450 resulting in a total number of 180 individuals, i.e., 60 per group. For the longitudinal part, a subset of
15 451 60 adult individuals (30 patients with Parkinson's disease and 30 patients with rheumatoid arthritis)
16 452 are selected, based on their ability and willingness to participate in the longitudinal part of the study
17 453 (12 months follow-up). The selected number of participants for the longitudinal study is based on
18 454 feasibility due to the complexity and high costs of the clinical trial. The total number of subjects in the
19 455 longitudinal study can be smaller, as each individual serves as their own control.
20 456

23 457 **Adverse events**

24 458 There are no major risks expected for participants. Minor common adverse effects of PF might include
25 459 headaches, nausea, insomnia, back pain, dyspepsia and fatigue[83]. Any occurring adverse events are
26 460 recorded at each visit in REDCap[65]. Serious adverse events are communicated to the study
27 461 coordinator and principal investigator within 24 h of their report.
28 462

31 463 **Data management, monitoring, analysis, and evaluation of data**

32 464 The study participants receive a study ID (pseudonym) which is used for all collected data. Self-
33 465 administered questionnaires are directly recorded in REDCap. Participant files are kept for at least 10
34 466 years at the respective clinical sites.

35 467 Weekly meetings between the study team, the different clinical partners, and the principal
36 468 investigator, ensure a close monitoring of the data. Any occurring adverse events or other issues are
37 469 thus handled immediately.

38 470 Different statistical tests are performed according to the nature of the data. A premature termination
39 471 of the study is not envisaged; therefore, no interim analysis is done. Different correlation measures
40 472 are applied, including Spearman correlation, mutual information on discretised data, distance
41 473 correlation, maximum information criterion, local similarity analysis and the bioenv approach.
42 474 Comparison across all omic levels allows identification of common and disease-specific signatures.
43 475 Multivariate machine learning is used to link different data features to observed patterns. For
44 476 additional confounding factors, especially in the cross-sectional study, multivariate statistical analysis
45 477 will be performed. These factors will be accounted for by including confounders in the analysis, e.g.,
46 478 as covariate in the statistical models.

47 479 The longitudinal part of the study continues for a period of 12 months. After finalisation of this period,
48 480 there is no follow-up of the participants. Interesting findings will be further validated using the existing
49 481 sample set and analyses may be performed on additionally collected samples.

50 482 The SPIRIT - checklist (Standard Protocol Items: Recommendations for Interventional Trials) was used
51 483 to write this protocol [84].
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484 Trial status

485 The recruitment for the ExpoBiome study started in April 2021 and is currently ongoing. All study
486 participants should be recruited by the end of 2022. The sample collection will take place from April
487 2021 to November 2023.
488

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493 Author contributions:

494 Study design and protocol were done by Bérénice Hansen, Cédric C. Laczny, Jochen G. Schneider,
495 Paul Wilmes; the interventional concept was drawn by Etienne Hanslian, Daniela Liebscher, Andreas
496 Michalsen, Anika Hartmann, Brit Mollenhauer, Sebastian Schade, Nico Steckhan, Jochen G.
497 Schneider, Paul Wilmes; the clinical trial was designed and is conducted by Etienne Hanslian, Daniela
498 Liebscher, Andreas Michalsen, Anika Hartmann, Brit Mollenhauer, Sebastian Schade; the procured
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503 Bérénice Hansen, Audrey Frachet-Bour, Janine Habier; the planning of the data analysis was done by
504 Cédric C. Laczny, Jochen G. Schneider, Paul Wilmes, Kirsten Roomp, Velma T.E. Aho, Marek
505 Ostaszewski; all authors contributed equally with edits, comments and feedback, read and approved
506 the final manuscript.
507

508 Ethics and dissemination

509 Ethical approval was obtained to plan and conduct the trial from the institutional review board of the
510 Charité-Universitätsmedizin Berlin (EA1/204/19), the ethics committee of the state medical
511 association (Landesärztekammer) of Hessen (2021-2230-zvBO) and the Ethics Review Panel (ERP) of
512 the University of Luxembourg (ERP 21-001-A ExpoBiome). The results of this study will be
513 disseminated through peer-reviewed publications, scientific presentations, as well as press releases
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517
518

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522

522 Competing interests statement

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Supplements

The SPIRIT checklist was used to write our report[84].

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26 731

732 Figure Legends

733 Figure 1. Study design. This figure illustrates the study design with five different arms in
734 total, two of which continue with the longitudinal part of the study. Visits take place at the
735 clinical sites at each timepoint and include the collection of the displayed samples. This
736 image was generated using Biorender software (<http://www.biorender.com>). T, timepoint;
737 W, week; D, day; M, month.

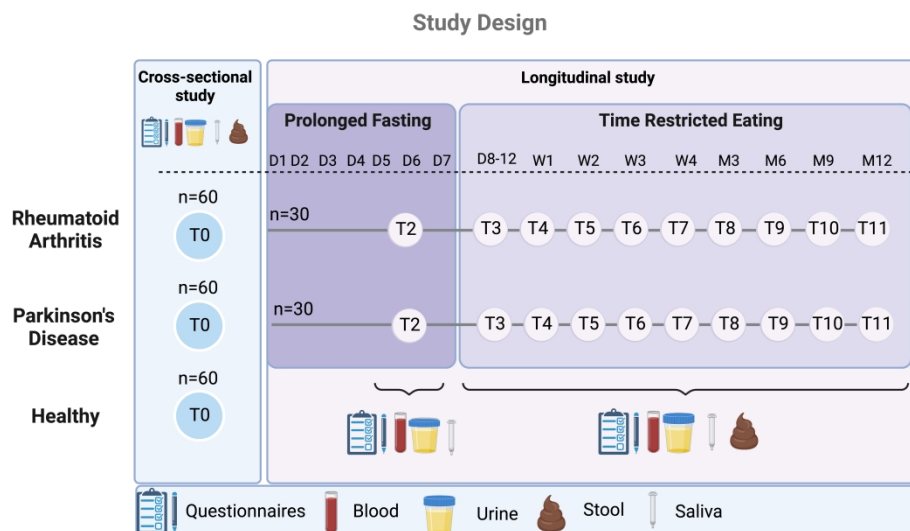


Figure 1. Study design. This figure illustrates the study design with five different arms in total, two of which continue with the longitudinal part of the study. Visits take place at the clinical sites at each timepoint and include the collection of the displayed samples. This image was generated using Biorender software (<http://www.biorender.com>). T, timepoint; W, week; D, day; M, month.

279x177mm (600 x 600 DPI)

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRIT reporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Gøtzsche PC, Altman DG, Mann H, Berlin J, Dickersin K, Hróbjartsson A, Schulz KF, Parulekar WR, Krleža-Jerić K, Laupacis A, Moher D. SPIRIT 2013 Explanation and Elaboration: Guidance for protocols of clinical trials. *BMJ*. 2013;346:e7586

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	n/a
Protocol version	#3	Date and version identifier	n/a
Funding	#4	Sources and types of financial, material, and other support	13
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
5				
6				
7				
8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	1, 13
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
13				
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15				
16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	1, 13
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
22				
23				
24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	5
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
31				
32	Background and	#6b	Explanation for choice of comparators	5
33	rationale: choice of			
34	comparators			
35				
36				
37	Objectives	#7	Specific objectives or hypotheses	5
38				
39				
40	Trial design	#8	Description of trial design including type of trial (eg, parallel	5
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
50				
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52				
53	Study setting	#9	Description of study settings (eg, community clinic, academic	6
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
56				
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1	Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
2				
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5				
6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	5,7
7	description			
8				
9				
10	Interventions:	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	5,7
11	modifications			
12				
13				
14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	7
16	adherence			
17				
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21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	6,7
22	concomitant care			
23				
24				
25	Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	10f
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34	Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	5
35				
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40	Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	12
41				
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45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	5
46				
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48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
52				
53				
54	Allocation: sequence	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be	n/a
55	generation			
56				
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provided in a separate document that is unavailable to those who enrol participants or assign interventions

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4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),
6			describing any steps to conceal the sequence until interventions
7	mechanism		are assigned
8			
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11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol
12	implementation		participants, and who will assign participants to interventions
13			
14	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial
15			participants, care providers, outcome assessors, data analysts),
16			and how
17			
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19			
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,
21	emergency unblinding		and procedure for revealing a participant's allocated intervention
22			during the trial
23			
24			
25	Methods: Data		
26	collection,		
27	management, and		
28	analysis		
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31			
32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and
33			other trial data, including any related processes to promote data
34			quality (eg, duplicate measurements, training of assessors) and a
35			description of study instruments (eg, questionnaires, laboratory
36			tests) along with their reliability and validity, if known.
37			Reference to where data collection forms can be found, if not in
38			the protocol
39			
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42			
43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,
44	retention		including list of any outcome data to be collected for participants
45			who discontinue or deviate from intervention protocols
46			
47			
48	Data management	#19	Plans for data entry, coding, security, and storage, including any
49			related processes to promote data quality (eg, double data entry;
50			range checks for data values). Reference to where details of data
51			management procedures can be found, if not in the protocol
52			
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54			
55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary
56			outcomes. Reference to where other details of the statistical
57			analysis plan can be found, if not in the protocol
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1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	12f
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	12f
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
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9				
10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	12f
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
18				
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21				
22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	n/a
23	interim analysis		including who will have access to these interim results and make	
24			the final decision to terminate the trial	
25				
26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	12
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
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33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	n/a
34			whether the process will be independent from investigators and	
35			the sponsor	
36				
37				
38	Ethics and			
39	dissemination			
40				
41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	2
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	2, 13
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
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53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	6
54			participants or authorised surrogates, and how (see Item 32)	
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1	Consent or assent:	#26b	Additional consent provisions for collection and use of	6
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
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6	Confidentiality	#27	How personal information about potential and enrolled	12
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
9				
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11	Declaration of interests	#28	Financial and other competing interests for principal investigators	13
12			for the overall trial and each study site	
13				
14				
15	Data access	#29	Statement of who will have access to the final trial dataset, and	12f
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
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20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	n/a
21	care		compensation to those who suffer harm from trial participation	
22				
23				
24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	2, 13
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
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33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	13
34	authorship		professional writers	
35				
36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
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41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	6
44	materials		participants and authorised surrogates	
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46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	7
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
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BMJ Open

Protocol for a multicentre cross-sectional, longitudinal ambulatory clinical trial in rheumatoid arthritis and Parkinson's disease patients analysing the relation between the gut microbiome, fasting and immune status in Germany (ExpoBiome)

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2022-071380.R3
Article Type:	Protocol
Date Submitted by the Author:	14-Jul-2023
Complete List of Authors:	<p>Hansen, Bérénice; LCSB Laczny, Cédric C.; LCSB Aho, Velma T.E.; LCSB Frachet-Bour, Audrey; LCSB Habier, Janine; LCSB Ostaszewski, Marek; LCSB Michalsen, Andreas; Charité Universitätsmedizin Berlin, Institute for Social Medicine, Epidemiology and Health Economics; Immanuel Hospital Berlin-Wannsee Branch, Department of Internal and Integrative Medicine Hanslian, Etienne; Charite Universitätsmedizin Berlin, Institute for Social Medicine, Epidemiology and Health Economics; Immanuel Hospital Berlin-Wannsee Branch Koppold-Liebscher, Daniela; Charité Universitätsmedizin Berlin, Institute for Social Medicine, Epidemiology and Health Economics; Immanuel Hospital Berlin-Wannsee Branch Hartmann, Anika; Charité Universitätsmedizin Berlin, Institute of Social Medicine, Epidemiology and Health Economics; Charité Universitätsmedizin Berlin, Department of Dermatology, Venereology and Allergology Steckhan, Nico; Charité Universitätsmedizin Berlin, Institute for Social Medicine, Epidemiology and Health Economics; University of Potsdam, Digital Health - Connected Healthcare, Hasso Plattner Institute Mollenhauer, Brit; University Medical Center Göttingen; Paracelsus-Kliniken Deutschland GmbH Schade, Sebastian; University Medical Center Göttingen; Paracelsus-Kliniken Deutschland GmbH, Roomp, Kirsten; LCSB Schneider, Jochen; LCSB; Saarland University Hospital and Saarland University Faculty of Medicine, Department of Internal Medicine and Psychiatry Wilmes, Paul; LCSB; University of Luxembourg, Department of Life Sciences and Medicine</p>
Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Immunology (including allergy), Rheumatology, Pharmacology and

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	therapeutics, Neurology, Evidence based practice
Keywords:	IMMUNOLOGY, Rheumatology < INTERNAL MEDICINE, MICROBIOLOGY, Parkinson-s disease < NEUROLOGY, NUTRITION & DIETETICS, Clinical trials < THERAPEUTICS

SCHOLARONE™
Manuscripts

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4 1 **Protocol for a multicentre cross-sectional, longitudinal ambulatory**
5
6 2 **clinical trial in rheumatoid arthritis and Parkinson's disease patients**
7
8 3 **analysing the relation between the gut microbiome, fasting and**
9
10 4 **immune status in Germany (ExpoBiome)**

11 5
12 6 Bérénice Hansen¹, Cédric C. Laczny¹, Velma T.E. Aho¹, Audrey Frachet-Bour¹, Janine Habier¹, Marek
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55 49
56 50 Word count: 4461

Abstract

Introduction

Chronic inflammatory diseases like rheumatoid arthritis (RA) and neurodegenerative disorders like Parkinson's disease (PD) have recently been associated with a decreased diversity in the gut microbiome, emerging as key driver of various diseases. The specific interactions between gut-borne microorganisms and host pathophysiology remain largely unclear. The microbiome can be modulated by interventions comprising nutrition.

The aim of our clinical study is to (1) examine effects of prolonged fasting and time-restricted eating (TRE) on the outcome parameters and the immunophenotypes of RA and PD with (2) special consideration of microbial taxa and molecules associated with changes expected in (1), and (3) identify factors impacting the disease course and treatment by in-depth screening of microorganisms and molecules in personalised HuMiX gut-on-chip models, to identify novel targets for anti-inflammatory therapy.

Methods and Analysis

This trial is an open-label, multicentre, controlled clinical trial consisting of a cross-sectional and a longitudinal study. A total of 180 patients is recruited. For the cross-sectional study, 60 patients with PD, 60 patients with RA and 60 healthy controls are recruited at two different, specialized clinical sites. For the longitudinal part, 30 patients with PD and 30 patients with RA undergo 5-7 days of prolonged fasting (PF) followed by TRE (16:8) for a period of 12 months. One baseline visit takes place before the PF intervention and 10 follow-up visits will follow over a period of 12 months (April 2021 to November 2023).

Ethics and dissemination

Ethical approval was obtained to plan and conduct the trial from the institutional review board of the Charité-Universitätsmedizin Berlin (EA1/204/19), the ethics committee of the state medical association (Landesärztekammer) of Hessen (2021-2230-zvBO) and the Ethics Review Panel (ERP) of the University of Luxembourg (ERP 21-001-A ExpoBiome). The results of this study will be disseminated through peer-reviewed publications, scientific presentations and social media.

Trial registration number at clinicaltrials.gov:

NCT04847011

Key words: Microbiome, fasting therapy, intermittent fasting, time restricted eating, chronic disease, rheumatoid arthritis, Parkinson's disease, nutrition, chronic diseases, ExpoBiome, inflammation, gut on a chip, HuMiX, immunophenotype, metagenomics, metatranscriptomics, metaproteomics, metabolomics

Strengths and limitations of the study

- The participants of the longitudinal study will be closely monitored for 12 months and routine blood parameters as well as anthropometric data and questionnaires will be precisely documented.

- 94 • This study will identify novel microbiome-derived common and disease-associated
95 molecules involved in immune system modulation in two major chronic diseases: RA
96 and PD.
- 97 • This study aims at also identifying novel targeted pathways to control chronic
98 inflammatory conditions in the future.
- 99 • A limitation is the heterogeneity of the cohorts regarding age and sex, which is due to
100 the prevalence of the diseases: RA is more common in women, while PD is more
101 common in men and has a later disease onset.
- 102 • A bias exists in choosing RA and PD as chronic disorders to study immunophenotypes
103 although generalisable results are targeted.
104

105 Introduction (1339)

106
107 The human microbiome is emerging as a key driver of various diseases through its complex of distinct
108 yet connected biomolecules (referred to as the “*expobiome*”)[1, 2]. The expobiome comprises a
109 diverse set of nucleic acids, polypeptides and metabolites which, in the gut alone, are present in
110 substantial concentrations[1]. However, the specific interactions between gut-borne microorganisms
111 and host (patho)physiology remain largely unknown. Although host genetics shape the composition
112 of the gut microbiome, the latter is particularly influenced by non-genetic factors such as lifestyle and
113 diet[3, 4]. Therefore, the microbiome is a plausible target to modify health outcomes.

114
115 Individuals suffering from chronic diseases, including autoimmune, metabolic, and neurodegenerative
116 diseases as well as cancer, often present alterations in their gut microbiome composition. These shifts
117 are typically characterised by an overgrowth of one or several microbial species with likely adverse
118 effects as well as a decrease in beneficial taxa[5]. Such imbalances are referred to as dysbiosis.
119 Although structural microbiome changes are clearly detectable, the mechanistic or functional
120 consequences of dysbiosis are still largely unknown. However, they may result in dysregulated
121 interactions with the immune system[6]. Considering the intricacy of the immune system, the
122 question arises whether the observed microbiome changes are cause or consequence of disease. This
123 implies that, in addition to the genetic predisposition of the host, the gut microbiome needs to be
124 considered a potential pathogenic factor or major driver of disease onset and course[3, 4].
125

126 RA and PD are two specific examples representing dysregulated microbiome-immune system
127 interactions [7, 8]. RA is a multifactorial, chronic, and systemic autoimmune disease, primarily
128 affecting the lining of the synovial joints with a higher risk and younger age for disease onset in women
129 and a global prevalence of 1%[9, 10]. The exact disease pathogenesis is still unclear and no cure for
130 RA currently exists. In addition to the common local articular symptoms of RA, systemic comorbidities
131 can affect the vasculature, metabolism and bones[11]. Besides various environmental risk factors e.g.
132 smoking and a Western diet, the host microbiome is associated with the pathophysiology of the
133 disease[12]. The diversity of the gut microbiome has been reported to be decreased in individuals with
134 RA, compared with the general population, and is correlated with disease duration, activity, and
135 autoantibody levels[13, 14]. Studies in murine models also report that autoimmune arthritis is strongly
136 attenuated under germ-free conditions[15]. The introduction of specific bacteria, e.g. segmented
137 filamentous bacteria, into germ-free animals or oral infection with *Porphyromonas gingivalis* drive
138 autoimmune arthritis through activation of T helper cells[15]. Several different taxa, including
139 *Prevotella copri*, *Lactobacillus* spp. and *Colinsella* spp. are enriched in the gut microbiome of patients
140 with RA and correlate positively with disease markers e.g. immunoglobulins IgA and IgG , while other
141 taxa like *Haemophilus* spp. and *Faecalibacterium* spp. are typically found at lower abundances in

1
2
3 142 patients with RA compared to healthy individuals[13, 16, 17]. Alterations of the gut microbiome may
4 143 therefore have an important impact on RA pathophysiology[12].
5 144

6 145 PD affects 0.4-2% of the population over 65 years worldwide and is the second most common
7 146 progressive neurodegenerative disease with men being 1.5 times more likely to be affected than
8 147 women[18]. Cardinal symptoms are motor deficiencies such as tremor and rigidity, but also include a
9 148 wide range of non-motor symptoms, such as hyposmia, depression, insomnia or cognitive impairment,
10 149 severely impacting patients' quality of life[19]. Aggregations of the protein α -synuclein in the
11 150 dopaminergic substantia nigra represent the main neuropathological manifestations[20]. PD-
12 151 associated loss of dopaminergic neurons involves mechanisms of inflammatory and autoimmune
13 152 responses with microglial activity as a major driver [21].Dysbiosis of the gut microbiome has been
14 153 associated with the characteristic motor deficits and pathophysiological changes in the enteric and
15 154 central nervous systems in animal studies. Increased relative abundances of the genera *Akkermansia*,
16 155 *Bifidobacterium*, *Lactobacillus*, and *Methanobrevibacter* and decreased abundances in
17 156 *Faecalibacterium* and *Roseburia* have been reported[22, 23]. Two recently published clinical-trials
18 157 with prebiotic supplementation in PD observed a shift in gut microbiome composition, an increase in
19 158 short-chain fatty acids (SCFA) and a reduction in non-motor-symptoms [24, 25]. Most patients with
20 159 PD suffer from gastrointestinal symptoms such as constipation and irritable bowel syndrome (IBS) -
21 160 like symptoms[26]. The gut-brain axis, e.g. by-products produced by the gut microbiome, may
22 161 contribute to the production of α -synuclein aggregates in the enteric nervous system[27]. In addition,
23 162 increased intestinal permeability[28] as driver for enteric inflammation occur in PD and substantiate
24 163 a role of peripheral inflammation in the initiation and the progression of the disease[29].
25 164

26 165 One factor with known major impact on the gut microbiome and on chronic diseases is diet[7]. Dietary
27 166 approaches as fasting have already been used by Hippocrates in the 5th century BCE and have been
28 167 applied ever since by numerous medical schools to treat acute and chronic diseases [30-32]. Various
29 168 practices of caloric restriction through fasting have repeatedly shown remarkable health benefits[33,
30 169 34]. Maifeld *et al.* found that a 5-day fast followed by a modified Dietary Approach to Stop
31 170 Hypertension (DASH), with additional emphasis on plant-based and Mediterranean diets, reduced
32 171 systolic blood pressure, BMI, and the need for antihypertensive medications at three months post
33 172 intervention compared with DASH alone [35].
34 173

35 174 Furthermore, Choi *et al.* demonstrated that cycles of a fasting-mimicking diet suppress autoimmunity
36 175 and stimulate remyelination via oligodendrocyte regeneration in a murine experimental autoimmune
37 176 encephalomyelitis (EAE) model[36]. Jordan *et al.* described a reduction in monocyte metabolic and
38 177 inflammatory activity after a short-term fast and conclude that fasting attenuates chronic
39 178 inflammatory diseases without compromising monocyte capacity for mobilisation during acute
40 179 infectious inflammation and tissue repair[37].
41 180

42 181 These improvements can, however, typically only be maintained for a limited period of time, and the
43 182 symptoms can reappear after reintroduction of the patients' standard diet. Hence, protocols to sustain
44 183 these beneficial effects are of utmost importance. In mouse models of PD, intermittent fasting (IF) has
45 184 led to several improvements including decreased excitotoxicity, reduced neurodegeneration and
46 185 protection against autonomic dysfunction, motor and cognitive decline[38].
47 186

48 187 IF and PF may have potent immunomodulatory effects which may partially be mediated by the gut
49 188 microbiome and the fasting induced alterations of the latter[39]. These microbial shifts include
50 189 upregulation of *Akkermansia muciniphila*, *Bacteroides fragilis*, other *Bacteroides* spp., Proteobacteria,
51 190 and butyric acid producing *Lachnospiraceae*, but also *Odoribacter*, which is negatively associated with
52 191 blood pressure[35, 40]. Interestingly, an overall decrease of the Firmicutes/Bacteroidetes ratio could
53 192 be observed, a high ratio is commonly associated with several pathologies, including RA [41].
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4 194 A potential mechanism underlying the observed beneficial effects induced by dietary interventions
5 195 might be a direct gut microbiome-immune system interaction by pattern recognition. The microbiome
6 196 can regulate the intestinal innate immune system by modulating toll-like receptor (TLR) expression on
7 197 immunosensor cell surface through microbe-associated molecular patterns (MAMPs), which can
8 198 consequently trigger cytokine production and up-regulation of molecules on antigen presenting cells,
9 199 leading to activation of T cells[42]. Therefore, a change in gut microbiome composition can lead to
10 200 different outcomes in immune signalling pathways and either favour or suppress inflammation and
11 201 autoimmunity.
12 202

13 203 The impact and importance of the gut microbiome on human physiology and its potential
14 204 modifications by nutrition and dietary patterns, have been underestimated for centuries[43]. Reasons
15 205 may include missing standardised therapeutic protocols, the interindividual variability in the response
16 206 to fasting, lack of knowledge about possible adverse effects, and difficulties in the interpretation of
17 207 underlying mechanisms seen in clinical trials, but also in the comparably low potential for achieving
18 208 economic revenue or scientific impact[8].
19 209

20 210 Modern experimental approaches and computational integration allow a multi-layer analysis of
21 211 digestive processes in low caloric settings including the gut microbiome[44]. These technological
22 212 developments also permit a closer investigation of the link between the immune system and severe
23 213 caloric restriction.
24 214

25 215 To our knowledge no clinical trials have been investigating the connection between IF or PF and PD in
26 216 humans so far[38]. Our study aims to elucidate the causal relationship between the gut microbiome
27 217 and the immune system. To do so, we will use analyses of the molecular basis of human-microbiome
28 218 interactions enabled by high throughput methodologies such as the combination of metagenomics,
29 219 metatranscriptomics and metaproteomics. Moreover, we are aiming at identifying new genes,
30 220 proteins, metabolites, and host pathways facilitating the development of novel diagnostic and
31 221 therapeutic tools[45, 46].
32 222

33 223 **Methods and Analysis (3122)**

34 224

35 225 **Study objectives**

36 226 The first objective of the study is to define specific gut microbiome-derived molecules in RA and PD,
37 227 compared to healthy individuals, and relate this information to the immunophenotypes of the
38 228 individuals. The second objective is to identify and track common and disease-specific molecular
39 229 signatures to predict the outcome of a gut microbiome-targeted therapeutic intervention, here
40 230 fasting, on inflammation-driven symptoms in RA and PD. The third objective of the study is to identify
41 231 and validate microbiome-derived effector molecules which downregulate pro-inflammatory innate
42 232 and adaptive immune pathways.
43 233

44 234 **Study design**

45 235 The ExpoBiome cohort consists of 180 adult individuals, meeting the exclusion and inclusion criteria
46 236 (Table 1), for the cross-sectional study (objectives 1 and 3) and 60 adult individuals for the longitudinal
47 237 study (objectives 2 and 3). There are five different arms in total: (1) RA – cross-sectional arm [60
48 238 patients], (2) PD – cross-sectional arm [60 patients], and (3) healthy controls – cross-sectional arm [60
49 239 patients], (4) RA – longitudinal arm [30 patients], (5) PD – longitudinal arm [30 patients] (Figure1).
50 240

At the first visit (T0), patients answer several questionnaires, and blood, urine, saliva, and stool samples are obtained (Table 2). The longitudinal arms (4) and (5) undergo a 5–7-day PF with a dietary energy supply of max. 350-400 kcal per day with vegetable or grain broths as well as fresh vegetable juices[31, 40]. After the PF, the longitudinal arms follow a dietary regimen including the concept of TRE for a period of 12 months following the 16:8 pattern[47]. This means that food intake is allowed ad libitum for 8 h, followed by 16 h of fasting where no food should be consumed. The intake of non-caloric beverages, e.g., water, unsweetened tea or coffee is, however, allowed. The participants attend one follow-up visit (T2) during the PF and 9 follow-up visits during the 12 months of TRE (Figure 1).

Patient and Public Involvement

Feedback of patients during former clinical trials at the study centre in Berlin was integrated in the planning and design of the fasting intervention of this study. Patients are not involved in the conduct, reporting, or dissemination plans of this research.

Recruitment and randomisation

Patients are recruited by the specialised sites via different sources, e.g., by direct referral from either a physician at the Immanuel Hospital Berlin and the outpatient department of the Institute of Social Medicine, Epidemiology and Health Economics at Charité-Universitätsmedizin Berlin, or the Paracelsus-Elena Clinic in Kassel, or by non-personal advertising strategies (e.g. flyers or social media). For PD, the patients are screened by an experienced movement disorders specialist for featuring at least two of resting tremor, bradykinesia, and rigidity according to the United Kingdom Parkinson's Disease Society Brain Bank criteria[48]. Additionally, patients must show evidence of a dopaminergic deficit, either with DaTScan imaging or with a clear response to dopaminergic drugs. Motor and non-motor symptoms are assessed with the MDS-UPDRS (part I – IV) including the Hoehn and Yahr (severity) scale[49]. Additional PD-specific scales as Parkinson's Disease Sleep Scale-2, Parkinson's Disease Questionnaire-39, Non-Motor Symptoms Questionnaire and Non-Motor Symptoms Scale are used.

For patients with RA, the diagnosis has been made prior to the study by an experienced rheumatologist according to the European League Against Rheumatism (EULAR) criteria[50]. All clinical stages of RA will be included. We excluded patients with a BMI <18.5, as this indicates underweight, and fasting is not recommended. We did, however, not include an upper limit as fasting might be especially beneficial for patients with a BMI >24.9 and more than 60% of patients with RA are classified as overweight or obese[51]. For comorbidities we excluded mainly diseases which are known to interfere with the gut microbiome and might be potential confounders.

The chosen exclusion criteria will optimize the pairing process of healthy controls and patients with either RA or PD. However, as we have two diseases with different anthropometric characteristics (including age, gender, BMI) and only one control group, adding additional inclusion and exclusion criteria in the recruitment process would compromise on optimized matching. Furthermore, for the longitudinal part of the study, each patient will serve as his/her own control over time. Participants meeting all the inclusion and no exclusion criteria (Table 1) are assigned to their respective groups (RA, PD, or healthy control) (Figure 1) for the cross-sectional study after written informed consent. Half of the patients from the RA group and half of the patients from the PD group is selected to take part in the longitudinal part of the study, including the fasting intervention according to their availability for all 11 visits and their willingness to follow TRE over 12 months. This study is an open-label trial, as blinding is not feasible in fasting interventions.

Table 1: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
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<ul style="list-style-type: none"> • Age 18-79 • One of the following diagnoses: rheumatoid arthritis (first diagnosis >6 weeks ago), Parkinson's disease OR healthy volunteer • Control ("healthy") individuals must be without any evidence of active known or treated RA, without any evidence of active, known or treated central nervous system disease, and without a known family history of idiopathic PD • Control individuals should match the RA or PD individuals as closely as possible (sex, age, education) • Present written declaration of consent • Ability to understand the patient information and willingness to sign the consent form • Consent to specimen collection and specimen use 	<ul style="list-style-type: none"> • Gout or proven bacterial arthritis • Participation in another study • Existing/current eating disorder (bulimia nervosa, anorexia nervosa) within the past 5 years • Severe internal disease (e.g. kidney deficiency with creatinine > 2mg/dl) • Existing vegan diet or fasting during the last six months • Presence or suspicion of atypical PD (e.g. early dementia, early autonomous dysfunction) • Diagnosis of chronic inflammatory bowel diseases, celiac disease or colorectal cancer according to the guidelines of the German Society of Gastroenterology • Use of anti-psychotic drugs • Antibiotic use during the previous 12 months • Start of novel therapy with disease-modifying anti-rheumatic drugs • Pregnancy or breastfeeding women • Contraindication for additional blood draws (e.g. haemoglobin <10) • BMI < 18.5 • Psychiatric illness that limits understanding of the examination protocol (unable to consent)
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290 Fasting dietary counselling

291 The fasting group is closely monitored by nutritionists trained in fasting therapy, backed up by
 292 physicians experienced in fasting, from the Charité – Universitätsmedizin Berlin and the Paracelsus-
 293 Elena Clinic to ensure a uniform implementation of the fasting guidelines and the well-being of the
 294 study participants. The monitoring consists of several in person and virtual meetings which held
 295 individually or in group settings. Five meetings including the visits T0 and T2 during the fasting week
 296 as well as a group meeting after PF to ensure a well-managed start to the TRE phase take place. Group
 297 sessions are standardised using a pre-set deck of slides to be discussed during the group meetings
 298 with only minor disease-related differences between the PD and RA groups. All longitudinal
 299 participants receive a study-specific script with information on fasting procedures. Although the
 300 adherence of the patients cannot be profoundly controlled in the ambulatory setting, the blood
 301 samples will allow us to have additional insight into the nutritional habits as well as the fasting state
 302 of the patients on the day of the visit (blood glucose levels).

303

304 Medication

305 The medical treatments of the patients are monitored and documented with every clinical visit. The
 306 fasting intervention might necessitate temporary adjustments of several medications e.g., anti-
 307 diabetic and anti-hypertensive drugs as insulin levels and hypertension will be reduced due to lack of
 308 food intake [31].

309

310 Data collection

311 Sample and data collection is performed at the two clinical sites, Charité – Universitätsmedizin Berlin
 312 and Paracelsus-Elena Clinic (Table 2).

313

314 *Table 2: Sampling procedures.*

a) Biochemical samples and procedures

Blood (123 mL at T0, 23 mL at T2-T11)

Stool collection (2 mL at T0 and T3-T11)

Saliva collection (3.5 mL at T0-T11)

Midstream urine (50 mL at T0 -T11)

315

b) Questionnaires

Disease specific

PD:

- Disease Activity Score (DAS-28) [52]
- Parkinson's Disease Sleep Scale-2 (PDSS-2) [53]
- Parkinson's Disease Questionnaire-39 (PDQ-39)[54]
- Simplified Disease Index Score (SDAI) [55]
- Funktionsfragebogen Hannover (FFbH-R) [56]
- Movement Disorder Society Unified PD Rating Scale (MDS-UPDRS)[57]
- Non-Motor Symptoms Questionnaire (NMSQ)[58]
- Non-Motor Symptoms Scale (NMSS)[59]

RA:

- Disease Activity Score (DAS-28) [55]

-
- Non-Motor Symptoms Questionnaire (NMSQ) [58]
 - Funktionsfragebogen Hannover (FFbH-R) [56]

Dietary behaviour and lifestyle

- Fasting experience, expectation, and intervention
- Lifestyle
- 24H-Food-recall
- Food Frequency Questionnaire (FFQ)

General health and well-being

- Health Assessment Questionnaire (HAQ)[60]
 - Bristol Stool Scale[61]
 - Quality of Life questionnaire (WHO-5)[62]
 - Hospital Anxiety and Depression Scale (HADS)[63]
 - Profile of Mood States[64]
-

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317

318 Anthropometric data and questionnaires

319 The electronic data capture system REDCap[65], a secure web-based application, is used to record all
320 individual specific data. All data is stored on a secure server infrastructure at the host institution in
321 Luxembourg. Weight, height, body mass index (BMI), heart rate and blood pressure in sitting and
322 standing position as well as waist-hip-ratio is determined at every visit. Dietary behaviour,
323 sociodemographic measurements (age, sex, education level, employment status, marital status),
324 family history, current and previous illness and co-morbidities, and current medications, as well as
325 disease-specific data, questionnaires about the well-being of the patients and data on the behavioural
326 factors are collected at baseline, T6 (week 3), T9 (month 6) and T11 (month 12) (Table 2).
327 Questionnaires (24h-Food Recall, Bristol Stool Scale) are answered at all visits by the study
328 participants. Data storage, analysis and exchange are done only in pseudonymised fashion. The
329 nutritional data is analysed using the Nutrilog 3.20 software (Nutrilog SAS, Marans).

330

331 Blood samples and parameters

332 Blood samples are collected at each visit, and immediately used for peripheral blood mononuclear cell
333 (PBMC) isolation (T0), analysis by the study laboratory and centrifugation to freeze plasma samples at
334 -80°C (T0-T11). A clinical standard laboratory report is generated after every visit for each study
335 participant (Table 3). In addition to routine blood parameters, anti-citrullinated protein antibody

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336 (ACPA), zonulin, fatty-acid binding protein 2 (FABP2), and calprotectin levels are measured. Aliquots
 337 are securely stored to account for novel observations and testing of hypotheses.

338
 339 *Table 3: Routine blood parameters measured at each timepoint (T0 for cross-sectional study, T0-T11 for longitudinal study)*

Haematology – EDTA- blood	Clinical Chemistry – Serum
Basophils, %	Albumin
Basophils, abs.	ALT, 37°C
Eosinophils, %	Alkaline Phosphatase, 37°C
Eosinophils, abs.	AST, 37°C
Erythrocytes	Bilirubin, total
Haematocrit	Cholinesterase
Haemoglobin	Cholesterol
HbA1c	Creatinine
Leucocytes	hs-CRP
Lymphocytes, %	Glucose, serum
Lymphocytes, abs.	Gamma-GT, 37°C
MCH	HDL-Cholesterol
MCHC	LDL-Cholesterol
MCV	Potassium
Monocytes, %	Sodium
Monocytes, abs.	Total Protein
Neutrophils, %	Triglycerides
Neutrophils, abs.	Uric Acid
Platelets	Urea/BUN
RDW	Proteins – Serum
Reticulocytes	Rheumatoid factor H 35.9
Reticulocytes	Hormones – Serum
Reticulocytes, abs.	Insulin
	TSH (basal)

340

341 Stool, urine and saliva samples

342 The samples listed in Table 2 are collected at each visit, except for stool samples on T2 (fasting week)
 343 and immediately frozen and stored at -80°C. Stool characteristics are recorded at the time of the
 344 sampling. Faecal samples represent the main sample type for resolving the dynamic processes driven
 345 by microbiome in the gut. Also, as the gut microbiome is prone to diurnal fluctuations, the stool
 346 samples are collected in the morning, as far as possible.

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4 348 **Methods applied to samples**

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6 349 7 350 **Biomolecular extractions**

8 351 The collected stool samples undergo a biomolecular extraction procedure to allow isolation of
9 352 concomitant DNA, RNA, proteins, peptides and metabolites from single, unique faecal water samples;
10 353 this process involves cryo-milling the samples in liquid nitrogen, disassociating metabolites from
11 354 membrane and cell wall components in a solvent mixture of methanol, chloroform and water and
12 355 lastly proteins and RNA extraction by a methanol/chloroform and phenol buffer [66, 67]. Faecal water
13 356 is recovered following centrifugation and filtration, at low-speed or low-flow, respectively, to avoid
14 357 cell lysis. Nucleic acids are preserved by the addition of ribonuclease inhibitors and isolated by silica-
15 358 column-based techniques. This protocol involves the use of a robotic platform, ensuring a higher level
16 359 of standardisation and reproducibility[2].

17 360

18 361 **Coupled metagenomic and metatranscriptomic analyses**

19 362 Prior to sequencing library preparation, internal standards are introduced to obtain quantitative
20 363 sequencing data[68]. Contamination-free metagenomic (MG) and metatranscriptomic (MT) data is
21 364 generated, processed and analysed using the Integrated Meta-omics Pipeline (IMP)[45], which
22 365 incorporates pre-processing, assembly, gene annotation, mapping of reads, single nucleotide
23 366 polymorphism calling, data normalisation as well as analyses of community structure and function in
24 367 a fully reproducible software framework based on Docker. The MG and MT data is specifically
25 368 screened for enrichments in genes and pathways with known immunogenic properties[69]. The
26 369 extracellular biomolecules are linked to specific microbial populations based on the intracellular
27 370 metagenomic data [70]. In addition, the sequencing data is mapped against genomes of food
28 371 components[44]. The quantitative data is also related to microbial population sizes to determine the
29 372 contribution of the resolved microbial populations in stool to the extracellular DNA and RNA
30 373 complements[71].

31 374

32 375 **Metaproteomics**

33 376 For the metaproteomic analyses, filtration is used to separate extracellular peptides from the obtained
34 377 (poly)peptides. The resulting smaller fractions are then desalted and analysed without proteolytic
35 378 digestion via liquid chromatography (LC) and mass spectrometry (MS) on an EasyNano-LC coupled
36 379 online to a QExactive-Plus mass spectrometer (ThermoScientific, Waltham, USA). The identification of
37 380 ribosomal peptides is done with an integrated catalogue of MG and MT data, while the non-ribosomal
38 381 peptides are identified using different tools, i.e., MyriMatch, DirecTag as well as CycloBranch[45, 72,
39 382 73]. The metaproteomic data also allows identification of extracellular (poly)peptides with possible
40 383 pathogenic functions including protein misfolding and molecular mimicry[74, 75].

41 384

42 385 **Metabolomics**

43 386 Metabolomic data is analysed using a combination of targeted and untargeted approaches [44, 67,
44 387 76]. This highlights the major metabolite classes produced by the gut microbiome with an effect on
45 388 human physiology including organic acids, SCFA, lipids, branched-chain fatty acids, branched-chain
46 389 amino acids, vitamins, bile acids and neurotransmitters. Besides external compound calibration series
47 390 for quantification and quality control samples to ensure data normalisation and data acquisition
48 391 quality assessment, the metabolite extraction fluid is fortified with multiple internal standards to
49 392 improve method precision and accuracy[77, 78]. The data is compared to in-house databases and
50 393 public mass spectral libraries to identify known metabolites. The metabolomic data complements the

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394 metagenomic and metatranscriptomic data and thus allows further establishments of conclusive links
395 to metabolic properties in the gut.

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397 Deep immune profiling

398 Deep immune profiling is done using a recently established and optimised panel of metal-labelled
399 antibodies together with cytometry coupled to mass spectrometry (MS), the Maxpar Direct Immune
400 Profiling System (MDIPA). This approach allows the simultaneous quantification of 38 parameters on
401 single cells. Whole blood is stained with the MDIPA kit and stabilised with Proteomic stabiliser Prot-1
402 (501351694, Smart Tube Inc., Las Vegas) before storage at -80°C. The quantified immune cells
403 included in the MDIPA panel are CD3+, CD4+, CD8+, monocytes, dendritic cells, granulocytes, MAIT, T
404 cells, NK and B cells[79]. Cytokine expression profiles is analysed on blood plasma using the Human
405 Luminex performance Cytokine Panel (R&D Systems Europe, Abingdon), measuring CCL3, CCL4, CCL5,
406 GM-CSF, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-18, IL-21, IL-27, IL-33, IFN- β ,
407 Galectin-1, IFN- γ and TNF- α [69].

408

409 Gut-on-a-chip models

410 PBMCs isolated from T0 blood samples are co-cultured with gut-derived microbes under
411 physiologically representative conditions using the gut-on-a-chip model HuMiX[80]. This model of the
412 human gastrointestinal interface allows the investigation of the interactions between immune,
413 epithelial and bacterial cells and specifically the response to fasting in personalised in vitro models.

414

415 The Expobiome Map

416 The Expobiome Map (<https://expobiome.lcsb.uni.lu>) illustrates the diverse complex of microbial
417 immunogenic molecules, including nucleic acids, (poly)peptides, structural molecules, and
418 metabolites. The interactions between this “expobiome” and human immune pathways are encoded
419 in the context of chronic diseases[1]. The ExpoBiome Map is visualised using the MINERVA
420 Platform[81]. Clicking on different elements on the map reveals factors they affect and are affected
421 by, allowing an easier navigation through the complex relationships between individual microbiome
422 components in relation to human disease. The multi-omics data generated in the present study will
423 be integrated with the Map.

424

425 Exploratory analysis of novel host-microbiome interactions

426 Unknown non-ribosomal peptides or metabolite features are associated through correlation with
427 transcripts, proteins, and metabolites. Extracellular DNA fragments, as well as transcripts, proteins
428 and ribosomal peptides are linked to their genomic context by using IMP[45]. The data generated by
429 the project will be connected and collated to existing, publicly available datasets.

430

431 Outcome parameters

432 Primary Outcome

433 The primary endpoint of the study is the characterisation of the gut microbiome. The evaluation
434 includes both between-group and within-group differences in the longitudinal study arms with the
435 fasting intervention.

436

437 Secondary Outcome Measures

438 Secondary outcomes include the identification of common and disease-specific molecular signatures
439 and the characterisation of microbiome-derived effector molecules impacting the innate and adaptive

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3 440 immune pathways. Furthermore, several additional parameters mentioned in *Anthropometric data*
4 441 *and questionnaires* are assessed over a period of 12 months.
5 442

7 443 **Sample size and power calculation**

8 444 A power calculation using pilot metatranscriptomic data based on faecal extracellular RNA samples
9 445 was performed to determine the number of subjects to be recruited for the ExpoBiome project. The
10 446 obtained relative abundances of genera were used for the calculation of the required sample size per
11 447 group. The power calculation was based on the algorithm as described by Tusher, Tibshirani, and
12 448 Chu[82]. To achieve a power of 90% (at $\alpha = 0.05$), a total of 50 individuals per group (RA, PD, healthy
13 449 controls) must be analysed. Considering any possible dropouts, 20% additional subjects are recruited,
14 450 resulting in a total number of 180 individuals, i.e., 60 per group. For the longitudinal part, a subset of
15 451 60 adult individuals (30 patients with Parkinson's disease and 30 patients with rheumatoid arthritis)
16 452 are selected, based on their ability and willingness to participate in the longitudinal part of the study
17 453 (12 months follow-up). The selected number of participants for the longitudinal study is based on
18 454 feasibility due to the complexity and high costs of the clinical trial. The total number of subjects in the
19 455 longitudinal study can be smaller, as each individual serves as their own control.
20 456

23 457 **Adverse events**

24 458 There are no major risks expected for participants. Minor common adverse effects of PF might include
25 459 headaches, nausea, insomnia, back pain, dyspepsia and fatigue[83]. Any occurring adverse events are
26 460 recorded at each visit in REDCap[65]. Serious adverse events are communicated to the study
27 461 coordinator and principal investigator within 24 h of their report.
28 462

31 463 **Data management, monitoring, analysis, and evaluation of data**

32 464 The study participants receive a study ID (pseudonym) which is used for all collected data. Self-
33 465 administered questionnaires are directly recorded in REDCap. Participant files are kept for at least 10
34 466 years at the respective clinical sites.

35 467 Weekly meetings between the study team, the different clinical partners, and the principal
36 468 investigator, ensure a close monitoring of the data. Any occurring adverse events or other issues are
37 469 thus handled immediately.

38 470 Different statistical tests are performed according to the nature of the data. A premature termination
39 471 of the study is not envisaged; therefore, no interim analysis is done. Different correlation measures
40 472 are applied, including Spearman correlation, mutual information on discretised data, distance
41 473 correlation, maximum information criterion, local similarity analysis and the bioenv approach.
42 474 Comparison across all omic levels allows identification of common and disease-specific signatures.
43 475 Multivariate machine learning is used to link different data features to observed patterns. For
44 476 additional confounding factors, especially in the cross-sectional study, multivariate statistical analysis
45 477 will be performed. These factors will be accounted for by including confounders in the analysis, e.g.,
46 478 as covariate in the statistical models.

47 479 The longitudinal part of the study continues for a period of 12 months. After finalisation of this period,
48 480 there is no follow-up of the participants. Interesting findings will be further validated using the existing
49 481 sample set and analyses may be performed on additionally collected samples.

50 482 The SPIRIT - checklist (Standard Protocol Items: Recommendations for Interventional Trials) was used
51 483 to write this protocol [84].
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484 Trial status

485 The recruitment for the ExpoBiome study started in April 2021 and is currently ongoing. All study
486 participants should be recruited by the end of 2022. The sample collection will take place from April
487 2021 to November 2023.
488

489 Ethics and dissemination

490 Ethical approval was obtained to plan and conduct the trial from the institutional review board of the
491 Charité-Universitätsmedizin Berlin (EA1/204/19), the ethics committee of the state medical
492 association (Landesärztekammer) of Hessen (2021-2230-zvBO) and the Ethics Review Panel (ERP) of
493 the University of Luxembourg (ERP 21-001-A ExpoBiome). The results of this study will be
494 disseminated through peer-reviewed publications, scientific presentations, as well as press releases
495 and social media postings (Twitter, LinkedIn). Study participants will be contacted and informed by
496 the respective clinical sites about the outcome and results of the study, once the data analysis has
497 been completed (dissemination phase).

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507 Liebscher, Andreas Michalsen, Anika Hartmann, Brit Mollenhauer, Sebastian Schade; the procured
508 funding was provided by Paul Wilmes; the planning of high-throughput applications, statistical
509 planning, sample size calculation and randomisation were defined by Cédric C. Laczny, Jochen G.
510 Schneider, Paul Wilmes, Kirsten Roomp; the initial draft of the manuscript and coordination of the
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512 Bérénice Hansen, Audrey Frachet-Bour, Janine Habier; the planning of the data analysis was done by
513 Cédric C. Laczny, Jochen G. Schneider, Paul Wilmes, Kirsten Roomp, Velma T.E. Aho, Marek
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521 Competing interests statement

522 None declared.

523 Supplements

524 The SPIRIT checklist was used to write our report[84].

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731 Figure Legends

732 Figure 1. Study design. This figure illustrates the study design with five different arms in
733 total, two of which continue with the longitudinal part of the study. Visits take place at the
734 clinical sites at each timepoint and include the collection of the displayed samples. This
735 image was generated using Biorender software (<http://www.biorender.com>). T, timepoint;
736 W, week; D, day; M, month.

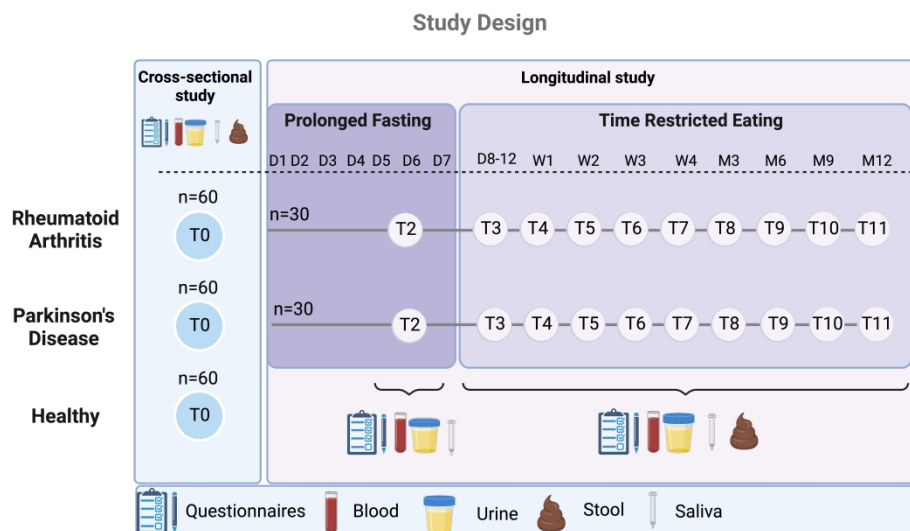


Figure 1. Study design. This figure illustrates the study design with five different arms in total, two of which continue with the longitudinal part of the study. Visits take place at the clinical sites at each timepoint and include the collection of the displayed samples. This image was generated using Biorender software (<http://www.biorender.com>). T, timepoint; W, week; D, day; M, month.

279x177mm (600 x 600 DPI)

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRIT reporting guidelines, and cite them as:

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		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	n/a
Protocol version	#3	Date and version identifier	n/a
Funding	#4	Sources and types of financial, material, and other support	13
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1

1	Roles and	#5b	Name and contact information for the trial sponsor	1
2	responsibilities:			
3	sponsor contact			
4	information			
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8	Roles and	#5c	Role of study sponsor and funders, if any, in study design;	1, 13
9	responsibilities:		collection, management, analysis, and interpretation of data;	
10	sponsor and funder		writing of the report; and the decision to submit the report for	
11			publication, including whether they will have ultimate authority	
12			over any of these activities	
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16	Roles and	#5d	Composition, roles, and responsibilities of the coordinating	1, 13
17	responsibilities:		centre, steering committee, endpoint adjudication committee,	
18	committees		data management team, and other individuals or groups	
19			overseeing the trial, if applicable (see Item 21a for data	
20			monitoring committee)	
21				
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23				
24	Introduction			
25				
26				
27	Background and	#6a	Description of research question and justification for undertaking	5
28	rationale		the trial, including summary of relevant studies (published and	
29			unpublished) examining benefits and harms for each intervention	
30				
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32	Background and	#6b	Explanation for choice of comparators	5
33	rationale: choice of			
34	comparators			
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37	Objectives	#7	Specific objectives or hypotheses	5
38				
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40	Trial design	#8	Description of trial design including type of trial (eg, parallel	5
41			group, crossover, factorial, single group), allocation ratio, and	
42			framework (eg, superiority, equivalence, non-inferiority,	
43			exploratory)	
44				
45				
46	Methods:			
47	Participants,			
48	interventions, and			
49	outcomes			
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53	Study setting	#9	Description of study settings (eg, community clinic, academic	6
54			hospital) and list of countries where data will be collected.	
55			Reference to where list of study sites can be obtained	
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1	Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
2				
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6	Interventions:	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	5,7
7	description			
8				
9				
10	Interventions:	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	5,7
11	modifications			
12				
13				
14				
15	Interventions:	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	7
16	adherence			
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21	Interventions:	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	6,7
22	concomitant care			
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24				
25	Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	10f
26				
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34	Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	5
35				
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40	Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	12
41				
42				
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45	Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	5
46				
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48				
49	Methods: Assignment			
50	of interventions (for			
51	controlled trials)			
52				
53				
54	Allocation: sequence	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be	n/a
55	generation			
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provided in a separate document that is unavailable to those who enrol participants or assign interventions

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4	Allocation	#16b	Mechanism of implementing the allocation sequence (eg, central
5	concealment		telephone; sequentially numbered, opaque, sealed envelopes),
6			describing any steps to conceal the sequence until interventions
7	mechanism		are assigned
8			
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11	Allocation:	#16c	Who will generate the allocation sequence, who will enrol
12	implementation		participants, and who will assign participants to interventions
13			
14	Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial
15			participants, care providers, outcome assessors, data analysts),
16			and how
17			
18			
19			
20	Blinding (masking):	#17b	If blinded, circumstances under which unblinding is permissible,
21	emergency unblinding		and procedure for revealing a participant's allocated intervention
22			during the trial
23			
24			
25	Methods: Data		
26	collection,		
27	management, and		
28	analysis		
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31			
32	Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and
33			other trial data, including any related processes to promote data
34			quality (eg, duplicate measurements, training of assessors) and a
35			description of study instruments (eg, questionnaires, laboratory
36			tests) along with their reliability and validity, if known.
37			Reference to where data collection forms can be found, if not in
38			the protocol
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43	Data collection plan:	#18b	Plans to promote participant retention and complete follow-up,
44	retention		including list of any outcome data to be collected for participants
45			who discontinue or deviate from intervention protocols
46			
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48	Data management	#19	Plans for data entry, coding, security, and storage, including any
49			related processes to promote data quality (eg, double data entry;
50			range checks for data values). Reference to where details of data
51			management procedures can be found, if not in the protocol
52			
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55	Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary
56			outcomes. Reference to where other details of the statistical
57			analysis plan can be found, if not in the protocol
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1	Statistics: additional	#20b	Methods for any additional analyses (eg, subgroup and adjusted	12f
2	analyses		analyses)	
3				
4	Statistics: analysis	#20c	Definition of analysis population relating to protocol non-	12f
5	population and missing		adherence (eg, as randomised analysis), and any statistical	
6	data		methods to handle missing data (eg, multiple imputation)	
7				
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10	Methods: Monitoring			
11				
12	Data monitoring:	#21a	Composition of data monitoring committee (DMC); summary of	12f
13	formal committee		its role and reporting structure; statement of whether it is	
14			independent from the sponsor and competing interests; and	
15			reference to where further details about its charter can be found,	
16			if not in the protocol. Alternatively, an explanation of why a	
17			DMC is not needed	
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22	Data monitoring:	#21b	Description of any interim analyses and stopping guidelines,	n/a
23	interim analysis		including who will have access to these interim results and make	
24			the final decision to terminate the trial	
25				
26				
27	Harms	#22	Plans for collecting, assessing, reporting, and managing solicited	12
28			and spontaneously reported adverse events and other unintended	
29			effects of trial interventions or trial conduct	
30				
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33	Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and	n/a
34			whether the process will be independent from investigators and	
35			the sponsor	
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38	Ethics and			
39	dissemination			
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41				
42	Research ethics	#24	Plans for seeking research ethics committee / institutional review	2
43	approval		board (REC / IRB) approval	
44				
45				
46	Protocol amendments	#25	Plans for communicating important protocol modifications (eg,	2, 13
47			changes to eligibility criteria, outcomes, analyses) to relevant	
48			parties (eg, investigators, REC / IRBs, trial participants, trial	
49			registries, journals, regulators)	
50				
51				
52				
53	Consent or assent	#26a	Who will obtain informed consent or assent from potential trial	6
54			participants or authorised surrogates, and how (see Item 32)	
55				
56				
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59				
60				

1	Consent or assent:	#26b	Additional consent provisions for collection and use of	6
2	ancillary studies		participant data and biological specimens in ancillary studies, if	
3			applicable	
4				
5				
6	Confidentiality	#27	How personal information about potential and enrolled	12
7			participants will be collected, shared, and maintained in order to	
8			protect confidentiality before, during, and after the trial	
9				
10				
11	Declaration of interests	#28	Financial and other competing interests for principal investigators	13
12			for the overall trial and each study site	
13				
14				
15	Data access	#29	Statement of who will have access to the final trial dataset, and	12f
16			disclosure of contractual agreements that limit such access for	
17			investigators	
18				
19				
20	Ancillary and post trial	#30	Provisions, if any, for ancillary and post-trial care, and for	n/a
21	care		compensation to those who suffer harm from trial participation	
22				
23				
24	Dissemination policy:	#31a	Plans for investigators and sponsor to communicate trial results	2, 13
25	trial results		to participants, healthcare professionals, the public, and other	
26			relevant groups (eg, via publication, reporting in results	
27			databases, or other data sharing arrangements), including any	
28			publication restrictions	
29				
30				
31				
32				
33	Dissemination policy:	#31b	Authorship eligibility guidelines and any intended use of	13
34	authorship		professional writers	
35				
36				
37	Dissemination policy:	#31c	Plans, if any, for granting public access to the full protocol,	n/a
38	reproducible research		participant-level dataset, and statistical code	
39				
40				
41	Appendices			
42				
43	Informed consent	#32	Model consent form and other related documentation given to	6
44	materials		participants and authorised surrogates	
45				
46				
47	Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of	7
48			biological specimens for genetic or molecular analysis in the	
49			current trial and for future use in ancillary studies, if applicable	
50				
51				

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