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The impact of nutrition and fasting on clinical phenotypes in rheumatoid arthritis

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I hereby declare that this dissertation entitled “THE IMPACT OF NUTRITION AND FASTING ON CLINICAL PHENOTYPES IN RHEUMATOID ARTHRITIS” has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than these indicated herein.

Esch-sur-Alzette, Luxembourg, 2024

Bérénice Hansen

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ABBREVIATIONS

Abbreviation	Meaning
24FR	24-hour food recalls
AB	Antibodies
ACPAs	Anti-citrullinated protein antibodies
ACR/EULAR	American college of rheumatology/European league against rheumatism
ADF	Alternate day fasting
ADP-PARP1	Adenosine diphosphate-pol(ADP-ribose) polymerase 1
AID	Activation-induced cytidine deaminase
AI	Anti-inflammatory
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
anti-CCP	Anti-cyclic citrullinated peptide
APCs	Antigen presenting cells
ATP/AMP	Adenosine triphosphate / Adenosine monophosphate
BCL-6	B-cell lymphoma 6 protein
BCR	B cell receptor
BTK	Bruton's tyrosine kinase
CD27	Cluster of Differentiation 27 (a protein found on some immune cells)
CD4+	CD4-positive T cells
CD40L	CD40 ligand
CDAI	Clinical disease activity index
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
csDMARDs	Conventional synthetic disease-modifying antirheumatic drugs
CVDs	Cardiovascular disease
CyTOF	Cytometry by time-of-flight
DAMPs	Damage-associated molecular patterns
DAS28	Disease activity score
DII	Dietary inflammatory index
DMARDs	Disease-modifying antirheumatic drugs
DMSO	Dimethyl sulfoxide
eCRF	Electronic case report form
EDTA	Ethylenediaminetetraacetic acid

EM	Extra-articular manifestations
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFA	Free fatty acids
FFbH-R	Hannover functional ability questionnaire
FFQ	Food frequency questionnaires
FLS	Fibroblast like synoviocytes
FMD	Fasting mimicking diet
FOXO1	Forkhead box O1
GCs	Glucocorticoids
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAQ	Health assessment questionnaire
HC	Healthy controls
HDL-C	High-density lipoprotein cholesterol
HIF-1α	Hypoxia-inducible factor 1-alpha
HLA	Human leukocyte antigen
HLA-DRB1	HLA class II DR Beta 1 chain
HSF-1	Heat shock factor 1
IBS	Irritable bowel syndrome
IF	Intermittent fasting
IgA	Immunoglobulin A
IGF	Insulin-like growth factor
IGF-1	Insulin-like growth factor 1
IgG Gc	Immunoglobulin G subclasses
IgM	Immunoglobulin M
iHuMiX	Human-microbial crosstalk
IL-1	Interleukin 1
IL-18	Interleukin 18
IL-6	Interleukin 6
ILD	Interstitial lung disease
Ir	Iridium
IRF-4	Interferon regulatory factor 4
JAK-STAT	Janus kinase-signal transducer and activator of transcription
K2E	Potassium ethylenediaminetetraacetic acid (EDTA) in K2 form

LDL-C	Low-density lipoprotein cholesterol
MAPK	Mitogen-activated protein kinase
MD	Mediterranean diet
mDCs	Myeloid dendritic cells
MDIPA	Maxpar® direct™ immune profiling assay™.
MHC	Major histocompatibility complex
miRNA	Micro-RNA
MMPs	Matrix metalloproteinases
mtDNA	Mitochondrial DNA
mTOR	Mechanistic target of rapamycin
mtROS	Mitochondrial ROS
MUFAs	Monounsaturated fatty acids
NAD+	Nicotinamide adenine dinucleotide (oxidized form)
NCDs	Non-communicable diseases
ND	Neurodegenerative disease
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3
NRF2	Nuclear factor erythroid 2-related factor 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAD	Peptidylarginine deiminase
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD	Pakinson's disease
pDCs	Plasmacytoid dendritic cells
PET	Polyethylene terephthalate
PF	Prolonged fasting
PGC-1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K-AKT	Phosphoinositide 3-kinase-AKT signaling pathway
PKA	Protein kinase A
PRR	Pattern recognition receptor
PUFAs	Polyunsaturated fatty acids
RA	Rheumatoid arthritis

RCP	Receptor interacting protein
REDCap	Research electronic data capture
RF	Rheumatoid factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCFA	Short chain fatty acids
SE	Share epitope
SF	Synovial fibroblasts
SGS	Société Générale de Surveillance SA
SIRT	Sirtuin
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SS	Sjögren's syndrome
ssDNA	Single-stranded DNA
SYK	Spleen tyrosine kinase
T2D	Type 2 diabetes
TCR	T cell receptor
Th1	T helper cell type 1
Th17	T helper cell type 17
Th2	T helper cell type 2
TLR9	Toll-like receptor 9
TMAO	Trimethylamine-N-oxide
TNFa	Tumor necrosis factor alpha
TRE	Time-restricted eating
Tregs	Regulatory T cells
TRF	Time-restricted feeding
tsDMARDs	Targeted synthetic disease modifying anti-rheumatic drugs
UNESCO	United Nations Educational, Scientific and Cultural Organization

SCIENTIFIC OUTPUT

Publication(s) in a peer-reviewed journal

- **Hansen B,** Laczny CC, Aho VTE, Frachet-Bour A, Habier J, Ostaszewski M, Michalsen A, Hanslian E, Koppold DA, Hartmann AM, Steckhan N, Mollenhauer B, Schade S, Roomp K, Schneider JG, Wilmes P. 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). *BMJ Open*. 2023 Aug 18;13(8):e071380. doi: 10.1136/bmjopen-2022-071380. PMID: 37597865; PMCID: PMC10441058.

- **Hansen B**, Roomp K, Ebid H, Schneider JG. Perspective: The Impact of Fasting and Caloric Restriction on Neurodegenerative Diseases in Humans. *Adv Nutr*. 2024 Apr;15(4):100197. doi: 10.1016/j.advnut.2024.100197. Epub 2024 Mar 1. PMID: 38432589; PMCID: PMC10997874.

Manuscripts under review in a peer-reviewed journal or in preparation

- **Bérénice Hansen,** Raul Da Costa, Fanny Hedin, Maria Konstantinou, Dominique Revets, Eduardo Jubal, Franck Ngangom, Cedric Laczny, Kirsten Roomp, Rajesh Rawal, Viacheslav Petrov, Etienne Hanslian, Daniela Koppold, Andreas Michalsen, Anika Rajput-Khokhar, Brit Mollenhauer, Sebastian Schade, Nico Steckhan, Michael Jeitler, Michel Vaillant, Antonio Cosma, Paul Wilmes, and Georg Jochen Schneider, *Mediators of Inflammation*, 2024, Immunophenotyping of patients with rheumatoid arthritis reveals difference in CD27+IgD+ unswitched memory B cell profiles.

- **Bérénice Hansen**, Rémy Villette, Viacheslav Petrov, Cédric C Laczny, Farhad Vahid, Kirsten Roomp, Etienne Hanslian, Daniela A Koppold, Anika Rajput Khokhar, Michael Jeitler, Nico Steckhan, Torsten Bohn, Sebastian Schade, Brit Mollenhauer, Andreas Michalsen, Jochen G Schneider, Paul Wilmes, *Nature Food*, 2025, Fasting-driven suppression of disease activity in rheumatoid arthritis

- **Bérénice Hansen,** Marta Sanchez-castro, Lynn Schintgen, Arefeh Khakdan, Jochen G. Schneider, Paul Wilmes, *Clinical Nutrition*, 2025, The impact of Fasting and Caloric restriction on Rheumatoid Arthritis in Humans: A narrative review.

Oral presentations at scientific conferences

- International Fasting Conference, Berlin, 23rd – 25th June 2023: Preliminary Metabolomic Data on Fasting in Parkinson's Disease and Rheumatoid Arthritis
- Life sciences Ph.D days 2023, Luxembourg, October 17th – 18th 2023: Fasting leads to disease activity suppression and sustained weight loss in rheumatoid arthritis

Teaching and science communication:

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- EuropaDonna, Munsbach, "Comprendre, prévenir et dépister le cancer du sein", Talk on Nutrition in breast cancer prevention, May 2023
- University of Luxembourg Open Days 2024, Luxembourg 18th May 2024: "Mikrobiom – wéi beaflosst dës onsiichtbar Welt eis Gesondheet?"
- University of Luxembourg BAMED 2024, Lecture on Nutrition and Health, May 2024
- ChdN, Wiltz, "Broschtkriibs – Preventioun, Diagnostik a Behandlung", Talk on Nutrition and lifestyle in breast cancer prevention, October 2024
- Luxemburger Wort, Intermittent fasting in elderly, Interview on intermittent fasting in October 2024

Poster presentations at scientific conferences (first author only):

- EMBL: The Spectra of Life, Heidelberg, December 7th – 9th 2022: Fasting dependent suppression of clinical activity of rheumatoid arthritis is associated with changes in the gut microbiome
- The 7th Venusberg Meeting on Neuroinflammation, Luxembourg May 11th – 13th 2023: Expobiome: A Multicentre Cross-Sectional, Longitudinal Study Analysing The Relation Between The Gut Microbiome, Fasting And Immune Status In Parkinson's Disease And Rheumatoid Arthritis Patients
- International Fasting Conference, Berlin, 23rd – 25th June 2023: The Effect of Fasting and Different Diets on Neurodegenerative Diseases: A Meta Analysis Review of Randomized Controlled Dietary Trials
- International Fasting Conference, Berlin, 23rd – 25th June 2023: A multi-centre cross-sectional, longitudinal trial on the relation between the gut microbiome, fasting and immune status in patients with Parkinson's disease and rheumatoid arthritis: the ExpoBiome study

- Nutrition 2023: Where the best in science and health meet The American Society for Nutrition (ASN), Boston, July 22th -25th 2023: Prolonged and intermittent fasting lead to disease activity suppression and sustained weight loss in rheumatoid arthritis
- Nutrition 2024: Where the best in Science & Health Meet, The American Society for Nutrition (ASN), Chicago, June 29th – July 2nd, 2024: Intermittent fasting leads to sustained weight loss without caloric restriction in patients with rheumatoid arthritis.
- Fondazione Prada, Human Brains, Milano, October 16th – 17th 2024: The Impact of Fasting and Caloric Restriction on Neurodegenerative Diseases in Humans

I. Abstract

Rheumatoid Arthritis (RA) is a chronic and systemic autoimmune disease affecting up to 1% of the global population. Prolonged fasting (PF) and Intermittent fasting (IF) have garnered attention for their potential health benefits in the treatment of RA suggesting a health benefit beyond the mere caloric restriction and weight loss. Some proposed underlying mechanisms suggest a role of the gut microbiome, ketone bodies, increased autophagy and DNA repair mechanisms amongst others. The improvements can, however, usaully only be maintained for a limited period and are reverted after reintroduction of the patients' standard diet.

The aim of this project was to elucidate the myriad of specific mechanistic factors between nutrition and the immune system that may help to sustain the beneficial effects of a fasting intervention on RA by tracking common and disease specific molecular signatures to predict the outcome of fasting on inflammation-driven symptoms in RA. The study was conducted in the framework of the ExpoBiome study, an open-label, multi-centre, controlled clinical trial consisting of a cross-sectional and a longitudinal study. 60 healthy controls and 60 patients with RA were recruited for the cross-sectional study, based on whose samples a detailed immunophenotyping was conducted. 30 patients with RA continued to the longitudinal study and followed a 5–7-day PF with a daily energy intake < 350 kcal. After the PF, they adapted a TRE pattern for a period of 12 months according to the 16:8 method with no additional dietary restrictions. By analyzing 24-hour food recalls (24FR) and food frequency questionnaires (FFQ), we aimed to discern any inadvertent dietary alterations. By integrating nutritional data with anthropometric measurements, routine blood chemistry and immunophenotyping, we aimed at elucidating the intricate mechanistic interplay between nutrition and the immune system, thereby potentially identifying underlying factors sustaining the beneficial effects of fasting interventions on RA.

The immunophenotyping performed on patients with RA compared to HC revealed a significantly reduced frequency of CD27+IgD+ unswitched memory B ($_{m}$B) cells in patients with RA (p-value < 0.01) compared to HC, with the disease RA being the primary and only significant factor explaining up to 17.9% of the variance of these cells. The fasting intervention induced a significant improvement in RA clinical disease activity index (CDAI) and wellbeing (HAQ, HADS, FFbH-R), beneficial changes in anthropometric factors such as decreased BMI and blood pressure, as well as improvements for several clinical parameters, including decreased glucose and cholesterol levels. A significantly improved CDAI and decreased BMI were observed for at least 12. The TRE was accompanied by several significant dietary shifts including a higher adherence to a mediterranean diet. Mixed models analysis revealed

that the observed improvements in RA disease activity were impacted by BMI, TRE and changes in the dietary composition.

We conclude that fasting, particularly PF followed by TRE, represents a promising, feasible, and sustainable strategy for prevention and management of RA without observed adverse side effects. This accessible intervention empowers patients to take an active role in symptom management through manageable lifestyle modifications. Also, observed reductions in BMI, cholesterol, blood pressure and glucose levels amongst others, suggest that fasting might offer broader applications addressing NCDs and could serve as valuable tool combating the global rise of NCDs.

II. Aims and Objectives

The main aim of the project was to investigate the impact of fasting and nutrition on RA to sustainably improve the health and wellbeing of patients with RA through nutritional interventions, namely PF and TRE, and decipher possible underlying mechanisms .

1. **Establish RA immunophenotype**: An in depth immunophenotyping of patients with RA, discerning them form healthy controls, was established serving as baseline to understand possible underlying mechanisms and to better understand the chronic and systemic autoimmune disease.

2. **Evaluate fasting intervention**: The immunophenotyping was followed by an assessment of dynamic changes in the interaction of different fasting strategies with several clinical aspects in patients with RA. The goal was to observe the effects of one week of prolonged fasting (PF) on RA and to analyse as to whether any beneficial effect could be sustained by implementing a time-restricted eating (TRE) strategy for 12 months.

3. **Analyse dietary habits**: In addition, dietary habits of the patients were recorded to detect any possible dietary changes and analyse the impact on patients with RA.

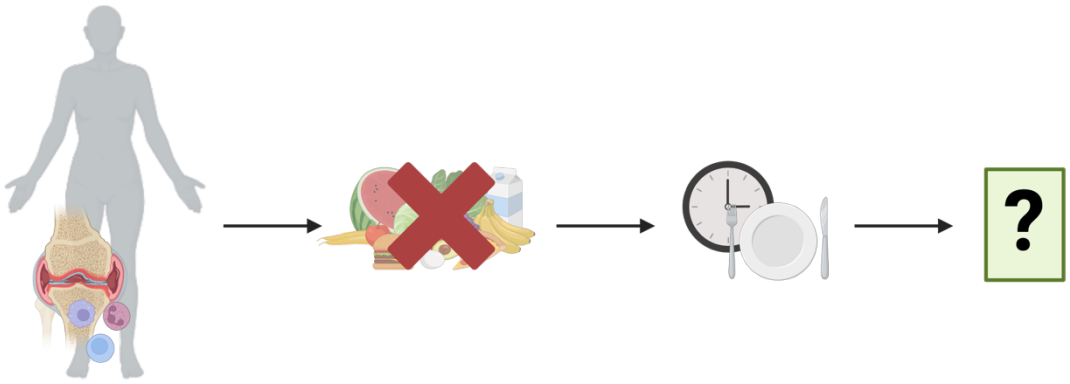

Figure 1: Aim of thesis project. Created in biorender. Hansen B, 2024

III. Materials and methods

The project of this thesis was mainly done in the framework of the ExpoBiome study. This is a multicentre, cross-sectional, and longitudinal clinical trial in patients with rheumatoid arthritis and Parkinson's disease. The Publication *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)* explains the study design in great detail in chapter V [1]. The thesis project included the different aspects of a clinical trial, preparing the E-Record in REDCap, planning and conducting the sample collection at different locations, on site preparation of samples and isolation of PBMCs for later processing, overcoming logistical challenges, planning the immunophenotyping experiments, further processing of the collected and stored samples, statistically analysing immunological data, evaluating of several different nutritional questionnaires and integration of clinical and anthropometrical data. The detailed experimental approaches have been explained and described in detail in the respective manuscripts in chapter V. Additional information on the different experimental processes will be elaborated hereafter.

A. First phase

The first phase of the research project was focused on the planning, initiation and conduction of the clinical trials in Germany:

1. REDCap: set-up of fully electronic FDA-conform eCRF system
2. Planning and organisation of study logistics including purchase and transport of consumables, legal documentation (Covid) and travel arrangements
3. Patient recruitment
4. Collection and preparation of all samples

This first phase is the focus of manuscript II in chapter V.

Experimental protocols

Plasma and serum for routine blood chemistry analysis by SGS

Plasma

1. Material
    a. BD Vacutainer K2E EDTA, 3 mL, 368856
2. Method

a. Blood withdrawal by study nurse.
b. Inversion of tubes.
c. EDTA-tube can be stored at RT until shipment to SGS (samples must be shipped on the same day as blood withdrawal).

Serum

1. Material
    a. BD Vacutainer Serum, 6 mL,366444
2. Method
    a. Blood withdrawal by study nurse.
    b. Wait for at least 30 min. after blood withdrawal before centrifugation to allow blood coagulation.
    c. Centrifuge 1300 - 2000 x g at RT, 10 min.
    d. Tube can be stored at RT until shipping to SGS (samples must be shipped on the same day as blood withdrawal has taken place).

Processing of plasma samples for cytokine analysis, isolation of PBMCs for iHuMiX, as well as stool, saliva and urine sample collection for gut microbiome analysis resp. follow-up experiments.

Plasma for Cytokine analysis

1. Material
    a. BD Vacutainer EDTA Plus tubes, 10 mL, PET, 367525
2. Method
    a. Blood withdrawal by study nurse.
    b. Inversion of blood tube.
    c. Centrifuge for 15 min., 1500xg, RT.
    d. Aliquot plasma in 4 x 2mL cryovials, store at -80°C.

PBMC isolation for iHuMiX

1. Material
    a. BD Vacutainer EDTA Plus tubes, 10 mL, PET, 367525
    b. Dimethyl sulfoxide (DMSO), D8418, Sigma-Aldrich, Germany
    c. Fetal Bovine Serum (FBS), A5669701, Gibco™ , Thermo Fisher Scientific
    d. Phosphate buffered saline (PBS), 14190144, Gibco™, Thermo Fisher Scientific
    e. OptiPrep Density gradient medium, 07820, Stemcell Technologies, USA
    f. SepMate™-50 (IVD), 85450, Stemcell technologies, USA

g. Freezing Container, Nalgene, Mr Frosty, C1562, Sigma-Aldrich, Germany

h. Trypan blue solution, 0.4%, 15250061, Gibco™, Thermo Fisher Scientific

2. Method

a. Prepare freezing medium:

i. Prepare 20% DMSO and 80 % FBS solution (put FBS in fridge day before).

ii. Sterilize: Take up with pipette, put into syringe, then put filter on.

iii. Put into fridge (can be stored for 1 working day).

b. Sepmate tube preparation:

i. Sample, medium (PBS) and density gradient medium must be at RT.

ii. Add density gradient medium to SepMate tube by pipetting through central hole of SepMate insert (some of the density gradient medium should be above insert).

c. PBMC isolation and freezing of cells:

i. Put whole blood into 4 x 50 mL falcon tubes, dilute with equal amount of PBS, mix gently.

ii. Add 30 mL of diluted sample per SepMate tube.

iii. Keep SepMate tube vertical, pipette sample along the side of the tube (not through hole).

iv. Centrifuge at 1200 g, 10 min., RT, acc 9 dec 9

v. Prepare cryovials and 7 x 50 mL falcon tubes.

vi. Discard supernatant above PBMCS, collect PBMC and put into falcon tubes (pour into tube).

vii. Fill up with 40 mL PBS.

viii. Wash 1: centrifuge 10 min, 300g, RT, 9acc, 9 dec

ix. Discard supernatant, add 20 mL PBS in one tube, dissolve pallet by pumping gently – take mix from one tube into next until all PBMCs from one patient in one tube.

x. Wash 2: centrifuge 10 min, 300g, RT, 9 acc, 9 dec

xi. Discard supernatant, add 40 mL PBS

xii. Wash 3: centrifuge at 10 min, 300 g, RT, 9 acc, 9 dec

xiii. Discard supernatant, dilute with 40 mL PBS

xiv. Count:

a. Take aliquot of 100 µL, dilute 1:5 (10 µL cells + 40 µL PBS) and 1:25

b. Dilute 1:2 each with trypan blue

xv. Wash 4: Centrifuge remaining sample again 10 min, 300 g, RT, 9 acc, 9 dec

xvi. Discard, add 1 mL FBS per $40*10^6$ cells, mix

xvii. Add same amount of DMSO+FBS freezing medium

xviii. Add 1 mL per cryovial (fast), put int cool cell box and place at -80°C asap.

xix. Place the freezing boxes or the Styrofoam container immediately into a -80°C freezer for 12 to 24 h (max 4 days), then transfer the cryovials into the liquid nitrogen tank.

xx. Avoid any temperature increase during the transfer in the nitrogen tank and, in general, prior to the thawing of the cells.

Stool samples

a. Material
   i. Sarstedt Screw Cap Tube (80.623.022)
   ii. Stool collector kit
   iii. Dry ice
b. Method
   i. Stool samples are collected by patient and immediately put on dry ice.
   ii. Samples are stored at -80°C.

Saliva

a. Material
   i. 2 mL Cryovials.
   ii. Bucket with dry ice.
b. Method
   i. Saliva is collected by patient into tube and immediately put on dry ice.
   ii. Samples are stored at -80°C.

Urine

a. Material
   i. 50 mL Sarstedt falcon tube
b. Method
   i. Urine is collected by patient into tube and immediately put on dry ice.
   ii. Samples are stored at -80°C.

B. Second phase

The second phase consisted in developing an experimental protocol and a gating strategy at the National Cytometry Platform of the Luxembourg Institute of Health to subsequently proceed with sample processing and data acquisition by CyTOF. The experimental phase was followed by supervised and unsupervised analysis of the data and appropriate statistical examination of the acquired data.

CyTOF combines the principles of flow cytometry with mass spectrometry to analyse single cells with high-dimensional accuracy. Cells are labelled with antibodies conjugated to metal isotopes rather than fluorophores. The process begins with the ionization of metal-labeled cells in a plasma torch. The resulting ion cloud is then analyzed by a time-of-flight mass spectrometer, which quantifies the metal isotopes attached to the antibodies. This generates high-dimensional data for each cell, encompassing information on multiple surface and intracellular markers[2].

This immunophenotyping is the topic of manuscript III in chapter V. The sample processing will be explained in detail hereafter. The protocol was established together with the NCP team.

1. Whole blood staining and stabilizing for CyTOF and Flow

a. Material

i. BD Vacutainer 4 mL, Natrium-Heparin, green (367869)

ii. MDIPA kit (Table in supplemental material of manuscript II chapter V), MDIPA kit, Standard Biotools, CA, USA

iii. Proteomic stabilizer Prot1, SmartTube Inc. San Carlos, CA, USA

b. Method

CyTOF staining and stabilization:

i. Within 30 min. (latest 8h after blood withdrawal - Fluidigm), aliquot 270 µL blood into a Fluidigm MDIPA antibody-tube (stored at 4°C).

ii. While adding the whole blood to the antibodies, gently mix while pipetting up and down.

iii. Incubate for 30 min. at RT.

iv. Add 420µl PROT-1 satbilizer (blood vs. Stabilizer ratio 1:1.4)

v. While adding PROT-1 to the antibodies, gently mix while pipetting up and down.

vi. Incubate for 10 min. at RT.

vii. Transfer total volume into labelled cryovial, freeze immediately at -80°C.

FLOW stabilization:

i. Aliquot 270 µL whole blood into three cryovials (3*270µL).
ii. Add 378µl PROT1 stabilizer into each cryovial (blood vs. stabilizer ratio 1:1.4)
iii. While adding PROT-1, gently mix while pipetting up and down.
iv. Incubate for 10 min. at RT, freeze immediately at -80°C.

2. Barcoding and intracellular staining of MDIPA stained whole blood sampées fixed with Smart Tube Prot 1 and stored at -80°C

a. Material

Buffer and Solutions

i. Ir-Intercalator, MDIPA kit, Standard Biotools, CA, USA
ii. MaxPar Fix and Perm Buffer, 201067, Standard Biotools, CA, USA
iii. Foxp3 Fixation/Permeabilization kit, 00-5523-00, invitrogen, MA, USA
iv. eBioscence Permeabilization buffer, 00-8333-56, Thermo Fisher Scientific
v. ddH_20, in house

Intracellular Antibodies Mix

i. BCL-6, REA373, 130-124-533, Miltenyi
ii. IRF4, IRF4.3E4, 646402, Biolegend

Sample preparation

i. MDIPA stained whole blood samples, MDIPA kit, Standard Biotools, CA, USA
ii. FA solution, Pierce, 16% Formaldehyde, 289006, Thermo Fisher Scientific
iii. PBS, 14190144, Thermo Fisher Scientific
iv. Barcode (Table in supplemental material manuscript III chapter V), Cell-ID, 201060, Standard BioTools, CA, USA
v. Maxpar Cell Acquisition solution PLUS (CAS PLUS), CAS PLUS, 201244, Standard Biotools, CA, USA
vi. Maxpar Four Elements EQ Beads, 201078, Standard Biotools, CA, USA

b. Methods

Buffer and Solutions

i. Ir-Intercalator (125 µM) is diluted in MaxPar Fix & Perm Buffer to a final concentration of 50 nM (Dilution 1/2.500).
ii. Prepare 1 ml of 50 nM Ir-Intercalator per 3 x 10^6 cells:
iii. Dilute 2 µl of the125 µM Ir-Intercalator aliquot in 18ul of Maxpar Fix & Perm buffer.
iv. Then, from the first dilution take 4ul to add in 1mL
v. eBioscience Foxp3 Fixation/Permeabilization working solution (1x):
   - part of Foxp3 Fix/Permeabilization Concentrate (4x)
   - parts of Foxp3 Fixation/Permeabilization Diluent

vi. eBioscience Permeabilization Buffer (1x) working solution:

- part Permeabilization Buffer (10x)
- 9 parts CyTOF ddH_20

Thawing protocol

i. 20 min prior to taking samples out of the freezer, precool the water bath at 12°C.

ii. Prepare AB mix and Thaw Lyse buffer 20mL (19.99mL of ddH2O + 10uL of Lyse Buffer) per aliquot and vortex. →200 mL (199,90 mL ddH2O + 100 µL Lyse Buffer) divided into 5 50 mL falcon tubes (5x 39,98 mL H2O + 20 µL Thaw Lyse Buffer)

iii. Thaw 10 aliquots (EDTA): Use 720µL per aliquot (i.e.: 300 uL WB + 420 uL PROT1)

iv. Thaw the aliquot for 6 mins at 12°C.

v. Transfer the thawed aliquot into a 50mL tube and add 4mL of the Thaw Lyse Buffer, incubate for 10 mins at RT.

vi. Centrifuge at 600g for 5 mins at RT, discard the supernatant and resuspend in 10mL of Thaw Lyse Buffer.

vii. Incubate for 10 mins at RT.

viii. Centrifuge at 600g for 5 mins at RT, discard supernatant.

ix. Add 5mL of CSB and transfer in a 15ml Falcon tube, centrifuge at 600g for 5 mins at RT.

x. Discard supernatant and resuspend cells in the residual volume.

Intracellular antibody staining

i. Add 1ml of 1x Foxp3 Fix/Perm buffer per 1 x 10^6 cells. Pipet up and down and incubate your samples for 45 min at 4°C. (3ml/sample)

ii. Without washing, add 2 ml of 1x Permeabilization buffer per 1 x 10^6 cells and centrifuge at 800g for 10 mins at 4°C. (6ml/sample)

iii. Remove supernatant.

iv. Add intracellular antibody mix (50 µL per tube)

v. Incubate for 30min at 4°C.

vi. Wash cells by adding 2 mL of CSB to each tube and gently vortex. Centrifuge tubes at 800 x g for 5 min, remove supernatant.

vii. Wash cells by adding 2 mL of CSB to each tube and gently vortex. Centrifuge tubes at 800 x g for 5 min, remove supernatant.

viii. Prepare a fresh 1.6% FA solution from the 16% formaldehyde stock ampule. Dilute 1 part of filtered stock formaldehyde with 9 parts PBS

ix. After the wash, discard the supernatant and add 1mL of the 1.6% FA solution to each tube and gently vortex to mix.

x. Incubate for 15 min at RT.

xi. Take the barcode tubes out of the freezer so they can thaw in the meantime.

xii. Centrifuge cells at 800 x g for 10 min.

xiii. Prepare 1mL of intercalator solution for each sample as described in a., and dissolve each barcode with 100ul of the intercalator solution.

xiv. After the centrifugation, discard the supernatant and resuspend cells in the residual volume.

xv. Add 900ul of the intercalator solution to each tube and resuspend the cells.

xvi. Add a barcode solution to each tube and mix gently the cells. Write down the barcode associated to each sample.

CyTOF sample preparation and acquisition
On the day of acquisition:

i. Wash cells by adding 2 mL of CSB to each tube and gently vortex. Centrifuge tubes at 800 x g for 5 min, 4°C.

ii. Wash cells by adding 2 mL of CSB to each tube and gently vortex. Centrifuge tubes at 800 x g for 5 min, 4°C.

iii. Pull the cells of each sample into a single tube. (first add resolved pellets in new tube, then wash tube with CAS + from next step and add in tube)

iv. Wash cells by adding 4 mL Maxpar Cell Acquisition Solution PLUS (CAS PLUS) to the tube and gently vortex. Centrifuge tubes at 800 x g for 5 min, 4°C.

v. Wash cells by adding 4 mL Maxpar Cell Acquisition Solution PLUS (CAS PLUS) to the tube and gently vortex. Centrifuge tubes at 800 x g for 5 min, 4°C.

vi. Carefully aspirate and discard supernatant. Gently vortex to resuspend cells in residual volume.

vii. Resuspend in 1mL of CAS PLUS.

viii. Filter the cells through a 35 µm cell strainer.

ix. Count the cells. Resuspend in CAS PLUS to reach 0.6 10^6 cells/ml.

x. Take 13.5 mL into 15 mL falcon tube, label with 100% volume (V=15 mL), store at 4°C

xi. Before acquisition, add 10% Maxpar Four Elements EQ Beads

3. Third phase

A third focus of the PhD project was to analyse the collected nutritional data and integrate this information with anthropometric, clinical and metabolomic data recorded during the cross-sectional and longitudinal clinical trial to examine the changes induced by PF and TRE in patients with RA. We collected nutritional data in food frequency questionnaires (FFQ), 24-hour Food recalls (24FR) and

questionnaires about dietary habits. The FFQ recorded the frequency and portions of consumptions of different food categories such as fruit, vegetables, legumes, cereals, dairy products, plant-based alternatives, meat, ultra processed foods, sweets, nuts, and herbs amongst others. In the 24FR the patients documented their food and drink consumption of 24 hours prior to the study visit. This detailed nutritional data was analysed by using the Nutrilog software and R studio for statistical analysis. The questionnaire focusing on dietary habits included detailed inquiries about dietary pattern, the number of meals consumed, the timing of these meals, the consistency of eating pattern, snacking habits, hours of overnight fasting, types of food consumed and eating hygiene amongst others. These questionnaires allowed us to get a deep insight into the dietary habits of the patients and to track their nutritional intake over the twelve months of the study. The nutritional and clinical data analysis is subject of manuscript IV in chapter V.

IV. Synopsis

A. A word on nutrition in health and disease

From hunting and gathering to the development of agriculture, domestication of animals, the industrial revolution and globalization, the access to food has changed dramatically over the history of humankind[3]. For most of our existence, we did not need to choose what to eat, we ate to survive.
However, health claims and nutrition recommendations have not been absent. Already Hippocrates (460 -375 BC) was convinced that diet and lifestyle were the source of many diseases, and his patients were often treated with strict vegetarian diets. Another well-known figure in the history of nutrition in health and disease is Otto Buchinger, a German physician born in 1878, known for his fasting practices, which will be elaborated in greater detail later.

Nutritional science has encountered numerous difficulties along the years, based on technical limitations and critical set-ups for clinical trials, ethical restrictions in research, reductionist approaches and growing impact of the food industry as well changes of the food items themselves, due to modern processing and environmental factors. Nonetheless, especially since the discovery of the importance of the gut microbiome and the role of metabolism in regulating the immune system, a clearer picture is emerging.

Today, as stated by the World health organization, we recognize nutrition as a critical part of health and development. Any form of malnutrition embodies a threat to human health. The double burden of disease results not only in nutrient deficiencies but also in several diet-related diseases[4]. To counteract this global increase in NCDs, we need to understand the diseases as well as the impact that lifestyle and especially diet has on them. High quality clinical nutrition intervention trials are indispensable and will provide key understandings paving the way to a holistic medical approach eventually improving global health.

This thesis aims at understanding the NCD RA and how the autoimmune disease can be modulated by applying different dietary strategies, in particular prolonged fasting and time-restricted eating, to achieve an improved wellbeing and a greater quality of life in patients with RA.

B. Rheumatoid arthritis

Rheumatoid arthritis is a systemic chronic autoimmune disease, attacking most prominently the synovial linings of the joints, affecting about 1% of the global population with women being at a threefold risk compared to men (Figure 2). Bone deformations named boutonnière or swan are commonly used in RA representations. The disease was first described by Augustin Jacob Landré-Beauvais in 1800. Despite over two centuries of research in RA, there is still no cure available. Patients suffer from a high burden of disease as RA does not only affect the joints but also comprises numerous comorbidities including interstitial lung disease, asthma, chronic obstructive pulmonary disease, thyroid disorders, osteoporosis and cancer[5]. Most respiratory symptoms manifest within the first 5 years of disease, but they might also precede the onset of joint symptoms in 10–20% of cases[6]. Additionally, RA increases the risk of cardiovascular disease (CVD), leading to a decreased overall life expectancy[7,5].

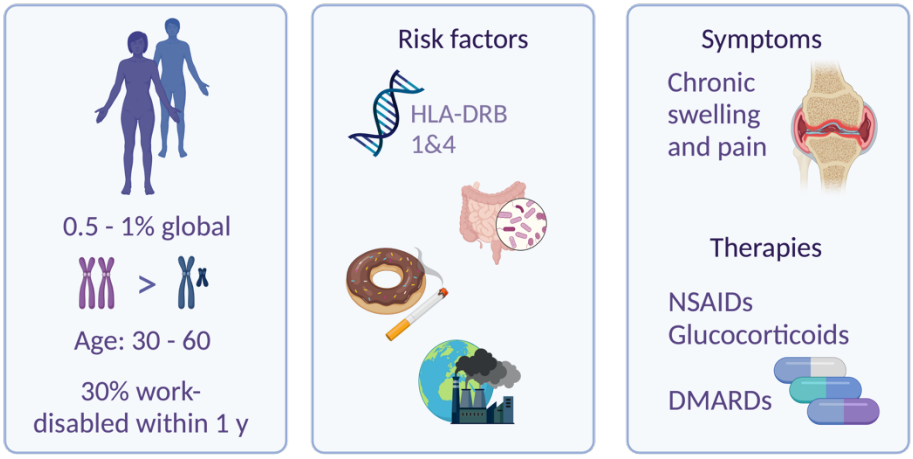

Figure 2: Rheumatoid arthritis overview.

The figure gives an overview of the disease prevalence, the age of disease onset, the most relevant risk factors, the most common symptoms and available drug therapies. Created in biorender. Hansen B, 2024

RA is typically distinguished by synovial inflammation and hyperplasia, autoantibody secretion, most commonly rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), and systemic manifestations involving various organs[8]. In addition to RF and ACPA, antibodies as anti-mutated citrullinated vimentin (MCV) against MCV have been identified as potential biomarkers in patients negative for ACPA and RF[8,9]. RA inflammation leads to pain classified as nociceptive, resulting from synovial inflammation and joint damage, including tendon binding, articular surface erosion, and localized osteoporosis[10].

The inflammatory process in RA involves multiple immune cells and cytokines, including IL-1, IL-6, IL-18, and TNF, with the NOD-like receptor protein 3 (NLRP3) inflammasome playing a central role as key source of IL-1 and IL-18[11].

Despite the fast-evolving research in RA, the underlying mechanisms of the disease are not clear yet. Several hypotheses have been proposed regarding the site of onset of the disease. It has been suggested that although synovitis is a hallmark of RA, the disease might start in the lungs where inhaled particles from smoking or polluted air can be citrullinated and subsequently lead to a break of tolerance of the immune system. A more recent hypothesis, the so called "mucosal origin", suggests that not only the lungs, but multiple mucosal sites, primarily gut, lung and oral, are involved in the onset of RA, each with distinct mechanisms[12].

Table 1: ACR/EULAR classification criteria for RA[13]

The 2010 ACR/EULAR classification criteria for RA (Total score >6 is considered satisfactory for the diagnosis of RA)	
Symptom	Score
Target population: Patients who a. Have at least 1 joint with definite clinical synovitis b. With the synovitis not better explained by another disease	
A. Joint involvement: • 1 large joint • 2- 10 large joints • 1 -3 small joints • 4 – 10 small joints • > 10 joints (at least 1 small joint)	0 1 2 3 5
B. Serology • Negative RF and negative ACPA • Low-positive RF or low-positive ACPA • High-positive RF or high-positive ACPA	0 2 3
C. Acute-phase reactants • Normal CRP and normal ESR • Abnormal CRP and abnormal ESR	
D. Duration of symptoms • <6 weeks • ≥6 weeks	0 1

1. Rheumatoid arthritis pathophysiology

RA evolves through different stages, typically divided into four main phases, each marked by distinct pathological and immunological changes. We distinct between susceptibility for RA, preclinical RA, early RA and established RA (Figure 3). The progression involves complex interactions between genetic predisposition, environmental factors and immune system dysregulation.

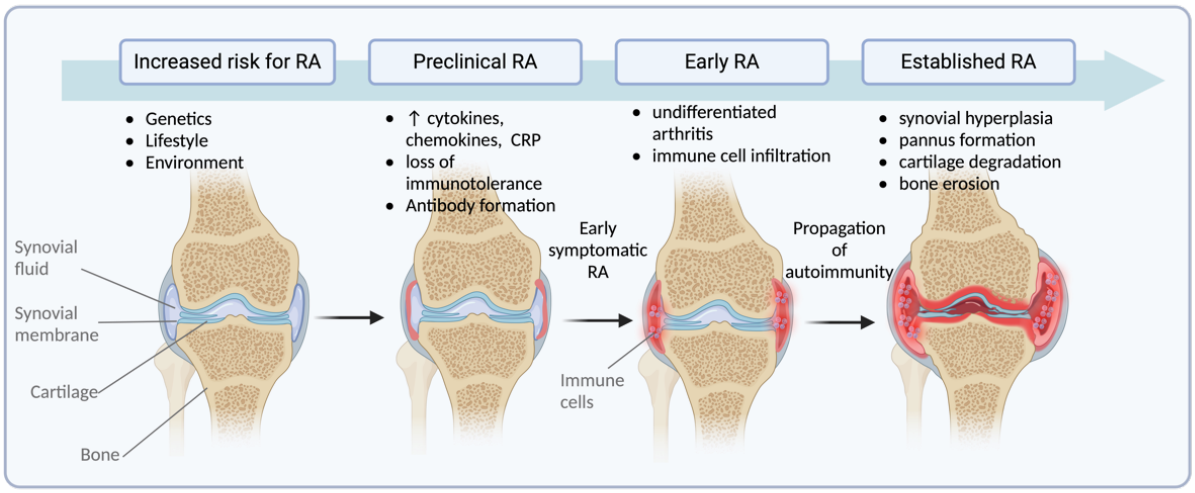

Figure 3: Stages of rheumatoid arthritis.

The figure shows the different stages of the disease RA starting at an increased susceptibility to develop RA based on the presence of several risk factors, moving into a preclinical stage with increased inflammatory markers and antibody formation and then going into stages of early RA and subsequently established RA with the hallmarks of RA as synovial hyperplasia, pannus formation, cartilage degradation and bone erosion13. Created in biorender. Hansen B, 2024

Susceptibility phase

The first stage does purely show a susceptibility for RA, no symptoms or abnormalities of the immune system can be detected at this point. This phase marks genetically predisposed individuals, where a combination of genetic and environmental factors, such as smoking and microbiome changes increase the risk for the development of RA[10,14]. Some of the susceptibility factors include specific genetic markers, such as the HLA-DRB1 shared epitope, which increases the likelihood of developing RA [10,14]. Several bacteria such as Porhpyromonas gingivalis have been suggested to be implicated in RA pathogenesis due to its production of peptidylarginine deiminase (PAD) enzyme possible inducing a citrullination of host proteins[15].

Preclinical rheumatoid arthritis

The preclinical phase is marked by asymptomatic autoimmunity, here patients begin to produce autoantibodies such as RF and ACPAs[14]. These ACPAs act against post-translationally citrullinated or

carbamylated proteins while RFs recognize glycation-modified IgG Fc fragments[14]. The post-translation modification processes are differently mediated, carbamylation is enzyme-independent while citrullination is an enzyme-mediated deamination of arginine[14]. This asymptomatic stage with dissemination of autoimmunity against altered self-proteins stage involves the systemic breakdown of self-tolerance, particularly towards the above mentioned post-translationally modified proteins[14]. Auto-ABs can be detected decades before clinical disease onset[8,14]. The immune response is not directed against native peptide sequences but against these modified proteins, with ACPAs binding to citrullinated peptides presented by HLA-DRB1 molecules and RF binding to citrullinated Fc region of Immunoglobulin G (IgG)[14,16]. It has been suggested that early steps of tolerance loss may occur in the lung and the gut with the gut microbiome being a potential risk factor[14].

Early rheumatoid arthritis

The transition to early RA is characterized by the onset of symptomatic synovitis and acute joint inflammation[14,17]. This stage is marked by the infiltration of immune cells, including CD4+ T cells, into the synovial membrane[14]. A significant aspect of this phase is the involvement of cell-intrinsic defects in CD4+ T cells, such as DNA repair defects, leading to telomere erosion and a hyperproliferative phenotype[14]. These defects facilitate the entry of immune cells into the synovial tissue[14,17]. T lymphocytes are polarized towards Th17 cells by the binding of citrullinated peptides to HLA-DRB1 molecules [16]. An imbalanced cytokine production by Th1/Th2/Th17 lymphocytes plays a key role in RA, as Th1 and Th17 are acting pro-inflammatory while Th2 has an anti-inflammatory function[16]. The synovial tissue in RA has been described as tertiary lymphoid tissue with two crucial pathogenic events of RA happening at this site of inflammation. An increased activity of macrophage like synoviocytes and FLS with increased cytokine and chemokine production as well as the infiltration of the synovial lining with adaptative immune cells mediating damage and bone erosion at a later timepoint[21]. Normally, FLS are responsible for the lining of the synovium, secretion of synovial fluid, lubrication of joint proteins and production of plasma protein for adjacent cartilage and joint cavity[10]. However, in RA, FLS undergo a transformation into aggressive, tumor-like cells that secrete inflammatory cytokines and matrix metalloproteinases (MMPs), exacerbating joint destruction[10]. Another important factor in cell differentiation, apoptosis and regulating immune function is the JAK-STAT pathway which is regarded as one of the central communication nodes in the functioning of the cell[18]. The occurrence and progression of RA have been associated with an abnormal activation of the latter, leading to an overproduction of pro-inflammatory cytokines, exacerbating immune dysregulation and driving disease progression[10]. During this early stage of RA, several factors are at work eventually leading to the chronicity of inflammation, including mitogen-activated protein kinase (MAPK) overactivation, closely correlated to articular cartilage destruction and hyperplasia, the PI3K (phosphatidylinositol 3 kinase)-AKT pathway, involved in the proliferation of FLS cells and synovial inflammation by stimulation cytokine expression, mTOR, inhibiting autophagy in FLS, several key players in B cell metabolism such as spleen

tyrosine kinase (SYK) and Bruton's tyrosine kinase (BTK), and Notch signalling pathways, affecting several steps of cell metabolism as proliferation, differentiation, and apoptosis[10,19].

Established rheumatoid arthritis

After the acute early phase of RA, as the disease progresses, a state of chronic established RA is reached. At this point we also see the important role of the adaptive immune system in RA as joint erosion is mainly induced by macrophages secreting pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1[21]. TNF can then further activate nuclear factor – κB (NF-κB) and MAPKs leading to an increased inflammation, drive osteoclastogenesis and inhibit osteoblast formation as well as Treg cell differentiation[19]. TNF has been proposed as the most important cytokine of the inflammatory cascade in RA as it is not only implicated in joint erosion and bone resorption as mentioned above, but also mediates the pannus formation via induction of endothelial cell activation as well as the induction of fever and pain via the PGE2 system[16]. Pannus refers to synovial hypertrophy and is a highly active tissue contributing to inflammation[20]. Thus, an uncontrolled TNF production is associated with RA. Also, IL-6 has been reported to be actively involved in the progression of RA by activation of endothelial cell, synoviocytes and osteoclasts[19]. Another cytokine implicated in RA pathogenesis is IL-1, acting as an endogenous pyrogen, inducing fever and supporting Th17 cell differentiation[19]. IL-1 as well as Th17 cells stimulate the production of receptor activator of NF-κB ligand (RANKL), leading to bone and cartilage destruction[19]. Other cytokines increased in the synovial fluid of patients include IL-17A and granulocyte-macrophage colony-stimulating factor (GM-CSF) acting in synergy to support chronicity and progression of RA[19]. The synergistic actions involve Il-17 increasing IL-1 and TNF, while IL-1 and TNF then activate IL-6 production (Figure 4). These effects happen in several cell types including chondrocytes and osteoblasts[19]. Another cytokine, IL-15 has been shown to induce severe inflammatory arthritis upon administration by promoting the differentiation of effector T cells and supporting B cell proliferation, leading to the synthesis of IgM, IgA and IgG in combination with CD40 ligand[21]. Different drug therapies specifically target the previously mentioned immunological factors and will be elaborated in greater detail below.

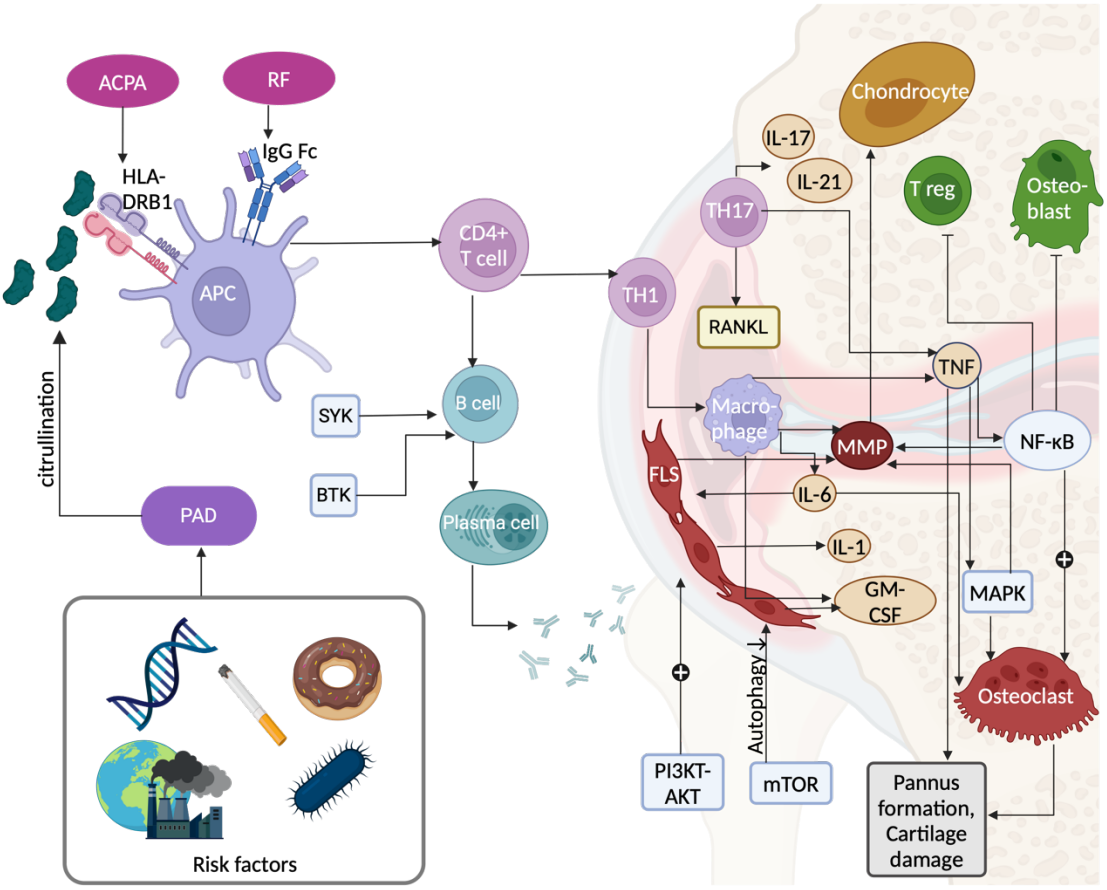

Figure 4: Pathogenesis of rheumatoid arthritis.

Several risk factors as genetics, smoking, diet, environment and the gut microbiome are implicate din RA disease onset and progression. The PAD enzyme is capable of citrullinating proteins, making them a target for anti-citrullinated protein antiboides (ACPAs). The rheumatoid factor recognizes glycation-modified IgG Fc fragments. Binding of different auto-antibodies leads to activation of several immunological cascades depicted in the figure, including an increased secretion of B and T cells, leading to an increased production of auto-antibodies and cytokines. This activation of the immune system eventually leads to the infiltration of the synovium, pannus formation and degradation of the cartilage. Created in biorender. Hansen B, 2024

1. Immunophenotype of rheumatoid arthritis

B cells

B cells play a major role in autoimmune diseases based on their capacity to present antigens as well as produce antibodies and cytokines. Together with T cells, they constitute the central components of the adaptive immune system and are continuously generated in the bone marrow. Each B cell develops a unique B cell receptor (BCR) which is made up of two heavy chains and two light chains. An immature B cell with an IgM marker on its surface exits the bone marrow into the bloodstream, migrating to the spleen. After several maturing steps, B cells are classified as naïve and are now able to be recognize antigens from pathogens[22].

To increase antigen affinity, B cells undergo secondary BCR diversification and accumulate mutations in their antigen-binding regions through the activity of activation-induced cytidine deaminase (AID)[22]. B cells can also undergo class-switch recombination, resulting in the expression of a different antibody class. Through the co-stimulation of the significantly upregulated CD40 ligand (CD40L) on T cell membranes, unswitched as well as switched B cells can further differentiate into memory B cells or plasma cells[22,23]. CD40L levels have been correlated with autoantibodies and disease activity in patients with RA[23]. Due to these antibody secreting capacities, B cells have been targeted by anti-CD20 B cell depleting drugs, such as rituximab, with promising effects[22]. Ever since the discovery of RF and ACAPs in RA, a key role of B cells has been appreciated and an enhanced B cell activity with a secretion of pro-inflammatory cytokines as IL-6 and TNF-α has been proposed [24,,22]. Overproduction of IL-6 has been observed in several autoimmune diseases, including SLE and RA[24]. The role of TNF-α in orchestrating peripheral blood memory B cell subsets in RA remains unclear. It has been reported that TNF-α directs memory B cells into inflamed tissues[25]. Additionally, TNF-α can induce cytokine production from unstimulated B cells without further antigenic stimulation, resulting in a pre-activated phenotype incapable of normal B cell function[24]. An accumulation of B cells in the synovial tissue and an overall higher B cell activation has been related to an increased activation of T cell and higher disease severity[24,26]. Unswitched memory B cells have been largely underappreciated and only recently emerged as a type of memory B cell with possible key roles in autoimmunity. The origin of these cells in the humoral system remains controversial[27]. One of their unique features includes a first line defense strategy through rapid secretion of low-affinity IgM upon pathogen challenge[28]. These unswitched memory B cells can be divided into two populations: IgM-expressing B cells and those that have lost IgM expression, becoming IgD+ only B cells through class-switch recombination[27]. They exhibit similarities with marginal zone B cells found in the spleen and have a unique phenotype. The BCR repertoire has been found to be altered in RA[29]. Unswitched B cells are amongst the major producers of natural IgM, which has been hypothesized to act protective in autoimmune diseases[29]. The natural IgM, as opposed to immune IgM, is produced in without apathogen encounter[29]. In addition to providing host defence against bacteria, as well as viral and fungal microbial infection, natural IgM has been suggested to be implicated in the clearance of apoptotic cells as well as neo-antigens, and suppressing the innate inflammation, supporting the immune homeostasis and protecting the body from autoimmunity[27].

Also, B cell-derived IgM can bind to various RA autoantigens, including ssDNA, fibrinogen, vimentin, and collagen. However, the B cells show dysfunctional features with reduced IgM secretion in patients with RA, which might play a role in disease pathogenesis[29]. Additionally, IgM from unswitched B cells in RA patients shows diminished binding to common RA autoantigens, indicating impaired IgM production[28]. In RA, studies on B cell abnormalities have produced varying results, with some indicating no change in peripheral memory B cells, while others report an increase. However, when it comes to unswitched memory B cells, decreased levels are observed in patients with RA as well as for conditions such as Sjögren's syndrome (SS), systemic lupus erythematosus (SLE), severe SARS-CoV-2 infections and malaria. This disturbance of B cell profile has

been linked to a possible disturbance in plasma cell differentiation[30]. The CD27 marker, which is a member of the TNF family, might play an important role in the generation of plasma cells producing autoantibodies in these autoimmune diseases[30].For SLE a reduced IL-10 production by unswitched memory B cells has been reported[27].

Also, the pattern recognition receptor (PRR) toll-like receptor 9 (TLR9) has been proposed to play a major role in the B cell differentiation by recognizing PAMPs and promoting B cell differentiation to $CD27^{high}$ via the CpG/TLR9 signal[28]. This subsequently facilitates the development of plasma blasts with high antibody producing capacity[28]. In addition, the TLR9 activation can indirectly influence mTOR signalling by changing the metabolic state of immune cells[28]. This DNA sequence typically found on membranes of bacteria and viruses also induces the differentiation from unswitched memory B cells to double negative CD27-IgD- memory B cells[28]. Unswitched B cells that have been stimulated with CpG secrete larger amounts of IgM and cytokines[28].

Overall, B cells play a crucial role in autoimmune disease and RA due to their different key functions as AB and cytokine production, antigen presentation and numerous intricate and incompletely understood implications in the adaptive immune system.

T cells in rheumatoid arthritis

RA is characterized by the predominant involvement of CD4+ T cells, which drive inflammation and autoantibody production within the synovial tissue. These synovial T cells, upon recognizing autoantigens, initiate and perpetuate inflammatory responses and are the major source of TNF, with the TNF-receptor 1 being expressed on synovial fibroblasts (SFs) and macrophages[14]. The well-known association between the HLA-DRB1 locus and RA underscores the influence of T cell selection and antigen presentation in autoreactive immune response induction[8]. Additionally, CD8+ T cell senescence in response to active viral antigens has been reported, suggesting a complex interplay between viral elements and immune responses in RA[31]. It has also been reported that T cell differentiation into T helper 1 cells is linked to the production of pro-inflammatory cytokines such as TNF-a and INF-γ, promoting inflammation and bone degradation. However, T cell involvement is not limited to Th1 and several studies have proposed a more complex involvement of T cells, including CD8+/CD4+ T cells immunosenescence, T regulatory action and the pro-inflammatory function of T helper 17 cells[31,32]. Th1 and Th17 cells, producing IFN-γ and IL-17 respectively, play critical roles in RA development. Pathogenic Th cells, which also produce GM-CSF (IL-22), are suggested to play a significant role in RA pathology. The reduction of Treg cells in RA may be partially mediated by IL-1β and IL-6-induced downregulation of Foxp3 expression. The plasticity of Tregs, Th1, and Th17 cells enables them to adapt functionally to various physiological conditions during immune responses through specific cytokine signaling. However, the precise functional roles of T cells in RA remain incompletely understood[8,33].

Immunophenotyping of the ExpoBiome cohort

Based on the complex interplay of different factors of the immune system in RA elaborated above, we decided to establish a baseline cross-sectional immunophenotyping of the patients with RA included in the ExpoBiome study compared to the included healthy controls. As RA is a dynamic disease with changing immunological phenotypes over the course of the disease, depending on the clinical disease activity amongst others, this baseline was of major importance to enable the subsequent studying and understanding of immunological shifts initiated during the targeted fasting intervention. The study design, the experimental protocols and the results of the immunophenotyping will be explained in detail in chapter V, manuscript III.

2. Risk factors to develop rheumatoid arthritis

Genetic and epigenetic factors

Several risk factors lead to the cumulative life risk to develop RA of about 3.6% for women and 1.7% for men. A genetic predisposition being the strongest risk, accounting for about 50-65% of disease occurrence[8,34-36]. The major histocompatibility complex (MHC) human leukocyte antigen (HLA) loci, such as HLA-DRB1, is strongly associated with RA[34]. The discovery of a highly preserved sequence of five amino acids in the DRß1-chain led to the proposal of the shared epitope (SE) hypothesis postulating the SE to enable binding of a specific peptide to the HLA region of antigen-presenting cells (APCs) with subsequent activation of an autoimmune response, leading to a more severe form of RA [37]. However, no such specific peptide has been identified until now and the SE hypothesis does not account for the involvement of other genetic factors [34]. The SE alleles might however facilitate the presentation of post-translational modified proteins, mostly by citrullination or carbamylation, leading to the production of autoantibodies (ABs) like anti-citrullinated protein ABs (ACPAs) [38]. Only about 30% of the genetic risk of developing RA can be explained by the MHC region and intensive research has led to the discovery of over 100 relevant different risk loci across multiple populations[8]. In 2021, 269 different single nucleotide peptides associated with RA were reported[39]. One of the non-HLA genetic variants representing a strong risk factor to develop RA is the PTPN22 gene, encoding a protein tyrosine phosphatase LYP which is expressed by the majority of the cells from the adaptive and innate immune systems [40]. Also, this gene locus could be regulating NLRP3-mediated IL-1ß secretion[41]. Single nucleotide polymorphisms (SNPs) play an important role in T and B cell development, resulting in T cell receptor (TCR) and BCR hyper-responsiveness [42]. Interestingly, gene variants of PTPN22 and HLA-DRB1 also modify intestinal microbiota composition, compromise granulocyte-mediated antibacterial defence in the gut, and reduce the suppressive effect of regulatory B cells and might be implicated in the gut dysbiosis observed in RA[43]. The role of the gut microbiome in RA will be further elaborated later in this chapter.

Thus, genetic susceptibility to rheumatoid arthritis is predominantly influenced by variations in the HLA-DRB1 gene, particularly the SE alleles, which play a central role in antigen presentation and autoimmune responses. Non-HLA genes, such as PTPN22, further contribute to the risk of developing RA by affecting immune cell signaling and regulation and are closely related to the occurrence of RA[10]. The consistency of

disease occurrence between monozygotic twins is only 12-15%, indicating an important role for other factors[8].

Gender and hormonal risk factors

Women are up to three times more affected by RA than men. This is a common observation in autoimmune diseases, although the underlying reasons are not clear. Hypotheses suggest that oestrogen is leaning to a pro-inflammatory effect while progesterone and androgens are acting anti-inflammatory[44]. In patients with RA, the ratio of oestrogens to androgens is significantly elevated in both male and female patients [45]. However, the results are conflicting and contradicting findings have been reported for breastfeeding, pregnancy and early menarche amongst others [34,46]. Some studies associate the increased prolactin levels to the increased risk of developing RA post-partum, however, the findings have been inconsistent and the rapid decline in progesterone might be another explanation for the increased risk of RA after childbirth [46]. The true effect of female reproductive hormones on the onset of RA is not fully understood.

Environmental and lifestyle risk factors

Smoking is recognized as one of the main extrinsic risk factors for RA [33]. The lungs have been found to be a major site of early pathogenic events and smoking has been reported to explain up to 25% of overall RA risk[47]. The hypothesis is that smoking is responsible for in-situ citrullination of proteins in the lungs of SE-positive patients, leading eventually to the break of immunotolerance and the subsequent generation of ACPAs [34]. Inhalation of other airborne agents as silica, textile dust and inorganic dust are associated with an increased risk for RA as well [8,34]. These environmental factors might not only be responsible for an increased citrullination, but also indirectly affect susceptibility genes via epigenetic mechanisms[10]. Epigenetic modifications are heritable although they do not involve alterations of DNA sequence but are mostly mediated by histone modifications, DNA methylation and noncoding RNA mechanisms[10]. MicroRNAs occupy an important position in modifying noncoding RNAs[10]. Epigenetics might explain the low correspondence of RA observed in monozygotic twins[48]. In addition, smoking has been found to be a mucosal microbiota modifier and it has been hypothesized that RA begins at the mucosal level before the disease transitions to the synovial tissue[33].

BMI is also a risk factor for RA although the exact underlying mechanism is not clear[33]. One hypothesis is that adipocytes secrete pro-inflammatory cytokines, adipokines amongst others, and contribute to an increase in estrogen[34]. Obesity has been associated with increased joint pain, especially in the knees, feet and hips as well as with a decreased time to RA occurrence[39,49]. Furthermore, sarcopenic obesity is associated with RA, even before the treatment with DMARDs[49]. Besides obesity, overweight and an increased weight circumference have also been linked to an increased risk for RA[39]. Adipsin is a fat derived factor that has been found to be a crucial link in arthritis pathogenesis and an adipsin deficiency was found to prevent joint infiltration with immune cells[50]. Adipose tissue is an essential player in the production of

pro-inflammatory mediators and is implicated in joint impairment and the chronic inflammation present in obesity[50]. Also myeloid cells were increased in the adipose tissue of patients with RA and are associated with systemic inflammation and autoimmunity and obese patients were found to have higher levels of inflammatory cytokines overall[33,41].

Lifestyle factors are important in both disease prevention and treatment which are unfortunately that historically have been greatly neglected and need to be addressed by both patients and treating physicians.

Oxidative stress and mitochondrial dysfunction

Oxidative stress plays a pivotal role in the pathogenesis of RA, contributing to the disease's inflammatory nature and progression. It is characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's ability to balance these reactive intermediates or repair the resulting damage[51]. This oxidative imbalance is implicated in triggering an intense immune response, including the activation of NLRP3 inflammasome, T cell differentiation and the release of inflammatory mediators such as NF-κB[41]. NF-κB is a key player in RA, promoting the release of cytokines and driving autoimmunity, which exacerbates systemic and local inflammation and contributes to synovial hyperplasia [41,49]. ROS can activate MMPs and therefore fuelling cartilage degradation in patients with RA[51]. Mitochondria, as central regulators of cellular metabolism and apoptosis, are deeply involved in the pathogenesis of RA through their role in oxidative stress[41]. Mitochondrial dysfunction is a significant contributor to RA, primarily due to its impact on cellular energy production and the subsequent generation of ROS but has also been suggested to modulate the inflammatory response of resident cells such as synoviocytes[41]. They generate ROS as by-products of the respiratory chain, particularly when there is an increased escape of electrons from the mitochondrial respiratory chain. This process not only contributes to oxidative stress but also leads to mitochondrial DNA (mtDNA) damage, protein oxidation, and lipid peroxidation, perpetuating a vicious cycle of mitochondrial damage and increased ROS production[41]. Some pathogens can negatively regulate mtROS generation and mtDNA sensing leading to suppression of first line defence mechanisms of the immune system. In the context of RA, oxidative stress and mitochondrial dysfunction have been closely linked to disease severity and activity[41]. Elevated mtROS levels are associated with higher plasma TNF-α levels, a key inflammatory cytokine in RA[41]. Treatments that block TNF-α have been shown to reduce oxidative stress, hypoxia-induced mitochondrial mutations, and overall disease severity, as measured by the Disease Activity Score (DAS28)[41]. Furthermore, oxidative stress in RA is exacerbated by the pro-inflammatory environment, where cytokines and other inflammatory mediators can further impair mitochondrial function, leading to increased ROS production and subsequent cellular damage[41]. The immune response in RA also involves the activation of the NLRP3 inflammasome, a critical component of the innate immune system that responds to cellular stress and damage signals[41,49]. NLRP3 is sensitive to mitochondrial-derived signals such as DAMPs, ROS, ATP, and oxidized mtDNA, which can activate the inflammasome and lead to the secretion of pro-inflammatory cytokines like IL-1β[41]. The inflammasome is also involved in Th2 and Th17 differentiation. This pathway is

particularly relevant in RA, where excessive inflammasome activation contributes to the chronic inflammatory state [41]. Additionally, hypoxia, a common feature in inflamed RA joints, further complicates the mitochondrial and oxidative stress landscape. Hypoxia can lead to the activation of hypoxia-inducible factor 1-alpha (HIF-1α), Notch and entail epigenetic modifications[41]. In RA, this adaptation often leads to a metabolic shift towards aerobic glycolysis, which is characterized by increased glucose consumption and lactate production. This metabolic reprogramming not only supports the energy needs of inflamed cells but also increases the production of ROS, further exacerbating oxidative stress and inflammation [41].

In summary, mitochondria and oxidative stress are central to the pathogenesis of RA. Mitochondrial dysfunction leads to increased ROS production, which contributes to oxidative damage, chronic inflammation, and disease progression. The interaction between oxidative stress, immune responses, and metabolic changes in RA highlights the complexity of the disease and underscores the potential benefits of therapies targeting these pathways to mitigate disease symptoms and progression [41,51].

Microbiome in rheumatoid arthritis

Changes in the mucosal microbiota have bene linked to RA and microbiome studies of bronchovascular lavage fluid of patients with early RA show a decreased diversity in the lung microbiota compared to healthy controls[33]. The genus *Pseudonocardia* was increased in patients with RA and mucosal-serum ratio of autoantibodies suggest that the lungs represent a primary site of autoantibody production[33]. Besides the lung, also the oral cavity, specifically periodontal disease, has been connected to RA[33]. Periodontitis has been associated with a higher risk to develop RA[8]. *Porphyromonas gingivalis* is found in periodontitis and is increased in ACPA positive patients. *P. gingivalis* has the ability to induce citrullination via PAD enzyme and might therefore play a role in RA initiation[33]. Also *Aggregatibacter actinomycetemcomitans*, promoting citrullination of neutrophil proteins, has been detected in the oral cavity of patients with RA[12].

The gut microbiome has been shown to be crucial for the establishment and maintenance of intestinal mucosa and a functioning immune system and has been linked to several pathologies in the past decade, including RA[33,52].It has been hypothesized that the inflammatory progress of RA intensifies dysbiosis, amplifying alterations. The gut microbiome varies largely between individuals and prevalences of each species can be affected by genetics, lifestyle, especially nutrition, drugs and other environmental factors. In healthy controls, the phyla *Bacteroidetes* and *Firmicutes* are commonly reported as the most prevalent[49]. When evaluating the gut microbiota of patients with RA, it is found to be altered, with an enrichment in gram-positive bacteria including *Eggerthella lenta*, *Clostridium asparagiforme*, *Lachnospiraceae* bacterium and *Gordonibacter pamelaeae* with *Prevotellaceae* representing the major association with dysbiosis in RA[53]. Interestingly, a peptide derived from *Prevotella copri* was shown to stimulate Th1 cell response when presented to HLA-DR[53]. In general, a reduction was observed for *Bacteriodes fragilis, Faecalibaterium, Fusicatenibacter, Bifidobacterium* and *Clostridium* genera amongst others[53]. However, the findings of changes in the gut microbiome composition of patients with RA have been contradictory and an increase in

Faecalibacterium prausnitzii , Collinsella and Bifidobacterium has been linked to an increased disease severity[54,55]. A Fecal microbiota transplants from patients with RA to mice increased the susceptibility to develop arthritis[33]. Also, the microbiome of patients undergoing etanercept treatment, shows a slight restoration, supporting the hypothesis of dysbiosis being a charactristic of the disease [56].

Besides the bacteriome also the mycobiome and virome should not be neglected in RA disease initiation and progression. Although thorough analyses of the fungi and gut viruses is lacking, an increased abundance of *Ascomycota* and decreased abundance of *Basidiomycota* has been found in the synovial fluid of patients with RA[53]. Regarding the virome, significant increases have been seen for *Streptococcaceae* phages and decreases for crAss-like phages amongst others[53].

While antibiotic treatments have been associated with a beneficial impact on disease severity and pro-inflammatory factors, they have also been associated with an increased risk to develop RA in a dose-response fashion[33,39].

Thus, the gut microbiome might be a crucial link in RA pathogenesis and will be further studied in subsequent analyses in the framework of the ExpoBiome study, these are however not part of this thesis[1].

3. Extra-articular manifestations and comorbidities in rheumatoid arthritis

Several extra-articular manifestations (EM) and comorbidities are associated with RA, including conditions such as rheumatoid nodules and vasculitis, as well as diseases affecting the neurological, gastrointestinal, and renal functions[57]. RA is also strongly associated with an increased incidence of CVD, which is the leading cause of death in RA patients[51]. The link between inflammatory processes in RA and atherosclerosis significantly contributes to the elevated CVD risk observed in these patients [57]. The underlying mechanisms contributing to this heightened risk include chronic inflammation, increased oxidative stress, and subsequent lipid peroxidation, all of which promote the development of atherosclerosis and related cardiovascular disorders [51]. Patients with RA are observed to have twice the risk of myocardial infarction and a 50% higher mortality risk from cardiovascular causes compared to the general population[57]. The primary clinical manifestations of CVD in RA include ischemic heart disease and congestive heart failure, which are reported to occur at approximately double the rate seen in the general population [57]. Furthermore, myocarditis has been detected in a notable percentage of RA cases during post-mortem examinations, with prevalence rates ranging from 11% to 50% [57]. The association between endothelial dysfunction and specific genetic markers, such as HLA-DRB1 SE, along with various inflammatory markers, further underlines the increased cardiovascular risk in RA patients [57].

The presence of ACPA is also linked to the development of CVD in RA patients, potentially leading to subclinical atherosclerotic changes [57]. Inflammatory cytokines, including TNF-α, IL-17, IL-6, and IL-1β, which are prevalent in RA and involved in the formation of pannus, contribute to endothelial cell activation and atherosclerosis[57]. A phenomenon known as the "lipid paradox" is observed in RA patients, characterized by lower levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein

cholesterol (HDL-C) during periods of high inflammation, which paradoxically coincides with an increased risk of CVD[58]. This paradox is partly explained by inflammation-induced alterations in HDL-C particles, which reduce their anti-atherosclerotic properties and promote LDL-C oxidation and plaque formation[58]. Dysfunctional HDL-C could worsen LDL-C metabolism abnormalities, thereby elevating the risk of cardiovascular disease, although the exact mechanisms remain unclear [58].

Several comorbidities affecting the gastrointestinal tract have also been reported as a direct consequence of RA, through related autoimmune diseases or a consequence of drug therapy[59]. Most reported is a liver dysfunction, affecting up to 50% of patients with RA followed by amyloidosis potentially leading to organ rupture or acute obstruction[57]. Mastication can be affected by Sjogren's syndrome leading to oral dryness, mouth ulcers or degraded temporomandibular joints[57].

Neurological EM can range from headaches, vertigo to meningitis or ischemic neuropathies due to necrotizing arteries[60]. Particularly the peripheral nervous system is consistently affected during RA progression and affects about 20% of patients[61]. Another pathology occurring as EM in RA is Alzheimer's disease and clinical trials have suggested that the use of TNF inhibitors or csDMARDs could reduce the risk of AD[57]. Other EM include renal disorders and increased malignancy for specific cancers as well as additional symptoms such as fatigue, mood disorders and fever amongst others[62].

4. Treatment in rheumatoid arthritis

Several treatment strategies have been developed over the past years and new drugs are being approved for RA treatment, including non-steroidal anti-inflammatory drugs (NSAIDs), conventional synthetic (cs), targeted synthetic (ts), biological (b) disease modifying drugs (DMARDs) and glucocorticoids (GCs)[63]. However, these drugs come with numerous side effects and many patients with RA are non-responders, even when administered high doses. The underlying causes are again unclear, multiple factors including amongst other genetics, environment, comorbidities, poor adherence and socioeconomics have been proposed[63,64]. In the context of this individual response and the topic of this thesis, a possible impact of fasting on medication and drug metabolism cannot be neglected. It has been reported that the latter might be modulated by an altered expression of involved enzymes[65]. Although the impact seems to be small, possible toxicity and increased side effects such as gastric damage caused by NSAIDs should be considered[65,66].

Non-steroidal anti-inflammatory drugs

NSAIDs are commonly prescribed for RA to alleviate pain and reduce inflammation, during acute flares achieved by inhibiting the synthesis of prostaglandins by suppressing cyclooxygenase activity and while being effective in symptom management, they do not alter the underlying disease process[10]. Long-term and/or excessive use of NSAIDs can lead to gastrointestinal, cardiovascular, and renal complications[10]. Also, some COX-2 inhibitors might increase adverse CVD effects by blocking cardioprotective prostaglandins and

overall NSAIDs may increase CVD risk by affecting blood pressure, coagulation processes and oxidative stress[63].

Glucocorticoids

Glucocorticoids, such as prednisone, offer potent anti-inflammatory effects and are often used for rapid disease control. They slow down radiographic progression but upon chronic use, they unfortunately come with numerous side effects, including ecchymosis, cushingoid feature, leg oedema, weight gain, depression, hypertension, suppression of hypothalamic-pituitary axis, increased risk for infections, osteoporosis and myopathy[10,67]. Long term use of high daily doses also increases the CVD risk in patients with RA[63]. Therefore, although glucocorticoids can be used during acute flares, they do not represent a sustainable solution for long-term disease control.

Conventional synthetic DMARDs

Methotrexate is the most used first-line, conventional synthetic DMARD reducing overall mortality by 60%[63]. The underlying mechanism involves dihydrofolate reductase, suppression of lymphocyte proliferation as well as secretion of pro-inflammatory cytokines[63]. By enhancing cholesterol transport in innate immune cells and improving endothelial function, methotrexate decreases the severity of atherosclerosis[63]. Other synthetic DMARDs include leflunomide, which inhibits pyrimidine synthesis, and sulfasalazine, with both anti-inflammatory and immunomodulatory effects.

Biological DMARDs

Biological DMARDs as Tocilizumab, Sarilumab and Anakinra are Il-6 or Il-1 receptor antagonists while Etanercept and infliximab act by blocking TNF activity. Despite their effectiveness, not all patients respond, and there is an increased risk of infections, including tuberculosis reactivation[10,19]. Memory B cells increased only in RF-negative patients during anti-TNF therapy, suggesting that the memory B-cells are more easily modulated in these patients[64].

Other bDMARDs as rituximab act as B-cell depleting, impacting antibody production and presenting antigens to T cells. Rituximab is particularly effective in patients who have not responded to TNF inhibitors[68].

T-cell co-stimulation inhibitors such as abatacept interfere with the activation of T cells, which are crucial in the pathogenesis of RA. However, as RA is a highly heterogenous disease, some patients respond only partially or not all to the treatments and remission is not achieved[33].

Targeted synthetic DMARDs

Targeted synthetic DMARDs, such as Janus kinase (JAK) inhibitors, represent a newer class of RA therapies[63]. JAK inhibitors as Tofacitinib or Baricitinib work by interfering with the JAK-STAT signaling pathway, which transmits signals from cytokine receptors on the cell surface to the cell nucleus, influencing DNA

transcription and immune cell activity[41]. Tofacitinib, has been shown to restore the balance between Tregs and Th17 by inhibiting the NLRP3 inflammasome[41]. The use of JAK inhibitors appears to be without severe side effects when used at approved doses, especially for patients with RA without certain risk factors[63].

Emerging therapies and adjunctive treatments

Besides the above mentioned and approved drugs available for RA, recent advancements have proposed several other acting mechanisms to relief RA symptoms and achieve disease remission. BTK inhibitors act by suppressing B cell receptor activation and osteoclast proliferation and might therefore offer promising effects in RA[8]. Other drugs focus on metabolic modulation, notably glycolysis, decreasing inflammatory response in macrophages and fibroblasts of patients with RA[41]. As chronic oxidative stress is a hallmark of RA, antioxidants have been shown to decrease both systemic and local oxidative stress, potentially slowing disease progression and mitigating tissue damage[51]. Also, the potential role of polyphenols as adjuvant therapies in RA is under investigation, given their anti-inflammatory properties, antioxidant activity, and ability to inhibit enzymes involved in eicosanoid production[51].

Although emerging treatments show promising avenues for better disease management and improved patient outcomes, there is a need for continued research and the development of therapies that are both effective and have minimal adverse effects. Lifestyle and nutritional intervention therapies as fasting or anti-inflammatory diets could offer strategies to prevent and treat RA in a sustainable long-term manner without compromising the overall health and wellbeing of the patients.

C. Fasting

Different forms of fasting have been practiced for centuries and can be observed in the animal kingdom, where hibernating mammals and migratory birds show immense long-term fasting capabilities[69]. During evolution, alternation of seasons and sunlight exposure-imposed differences in food availability and scarcity[70]. Religion and tradition showcase many examples of fasting, with the Lent in Christianity and Ramadan in Islam being some of the most prominent ones[69]. Importantly, fasting differs from starvation by voluntarily abstaining from eating with the purpose of a repair and regeneration function[69]. Also, fasting differs from and does not necessarily imply caloric restriction (CR). CR is defined as a reduction in energy intake by approximately 30%, while avoiding any nutrient deficiencies. During intermittent fasting (IF) the overall energy intake is not automatically reduced. IF is an umbrella term including various strategies such as alternated day fasting (ADF), with no or very little energy intake every other day, 5:2 fasting, with food consumption on 5 days and no or very little energy intake on 2 days, and time-restricted eating (TRE) where the food consumption is restricted to a specified eating window per day. TRE does not require a restriction in energy intake. The focus of this form of IF is the extension of the overnight fasting window as well as the consistency of an eating pattern. Both aspects have been proposed to offer systemic health benefits superior to CR alone. For other fasting types such as prolonged fasting (PF) and fasting-mimicking diets (FMD), CR is part of the protocol. PF can last up to several weeks and usually only allows for an energy intake of up to 350 kcal/day. PF is defined as a fast lasting longer than 4 days. Several different forms of PF have been used, including water only fast. Another popular option is Buchinger fasting, according to the protocol developed by the German physician Otto Buchinger, known as pioneer of medical fasting. Suffering from severe polyarthritis, he successfully underwent a PF in 1919 for 19 days[71] and subsequently published "The Therapeutic Fasting Cure" in 1935, which is still considered an important milestone for fasting nowadays. During the Buchinger fasting, up to 350 kcal/d can be consumed in the form of vegetable broth or juice, occasionally with the addition of honey[72] (Figure 5).

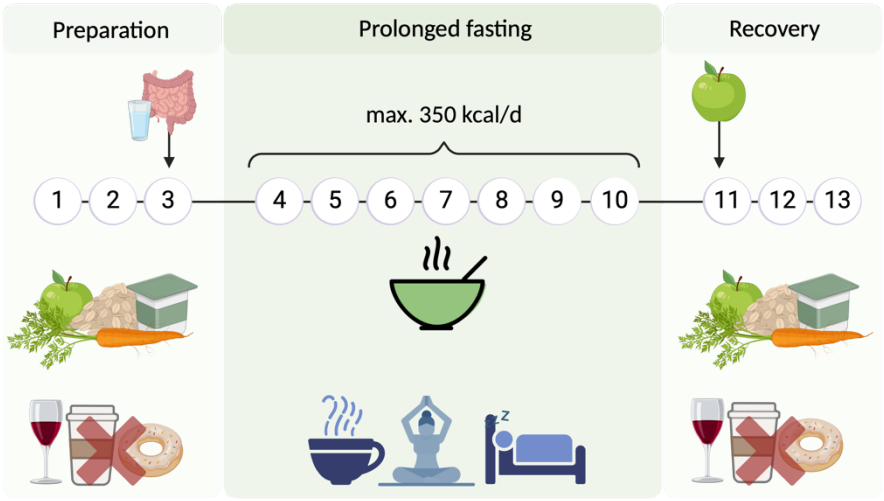

Figure 5: Buchinger fasting protocol.

The Buchinger fasting starts with up to three preparation days, slowly decreasing kcal intake and moving to an easy digestible diet, high in carbohydrates and low in fat and proteins, excluding alcohol, coffeine and junk food. The prolonged fasting is initiaed with a bowel cleanse and usually lasts 7-10 days during which the patients are advised to consume a max. of 350 kcal per day, while taking in enough fluids (water, herbal tea) and incorporating some light movement and enough sleep. After the completion of the fasting period, the fast is broken with the consumption of the first meal, starting the recovery phase. During these days, the patients slowly start to reintroduce solid food and increase the overall kcal intake. Created in biorender. Hansen B, 2024

One case report of this form of PF has been published in 2024, reporting a man who has fasted yearly for three weeks for 45 years. His biological age was 5.9 years younger than his chronological age[69]. Another publication describes the case series of 14 patients suffering from long Covid, undergoing supervised long-term fasting. The results show that only one patient did not experience beneficial changes, while for the other patients both frequent and less frequent symptoms were improved[73]. Additional beneficial effects of PF have been reported such as weight loss, a reduction in abdominal circumference and blood pressure, modulating effects of blood lipids and blood glucose as well as an increase in physical and emotional well-being and substantial pain relief in patients with chronic pain[74-76]. Although previous studies have shown a reduction in weight during fasting interventions alongside CVD improvements, weight-loss-independent benefits have also been proposed. Amongst these figure increased BHB, human growth hormone and haemoglobin as well as enhanced natriuresis and autophagy, increased immunity and gut microbiome eubiosis[77-79]. Although fasting has been practiced for generations, its underlying mechanisms and health effects are not exactly understood and the current research focuses on its impact on longevity, health span expansion, multi-stress resistance, antioxidant defence stimulation, improved immunity as well as prevention or treatment of NCDs[70].

Despite the many benefits that have been observed in PF, this dietary intervention does not come without its challenges, including risk for malnutrition and difficult implementation for several patients[78]. Therefore, different fasting strategies as IF, TRE or FMD have gained in popularity as they show similar promising effects on health while being more user friendly and carrying less risks for nutrient deficiency, muscle loss or typical fasting side effects as headaches or nausea.

Table 2: Different types of fasting[70,78,80]

Type of fasting	Duration / re-occurrence	Energy intake
Prolonged fasting / Long-term fasting	> 4 days – several weeks	200 – 350 kcal / day
Short-term fasting	2-4 days	200 – 350 kcal / day
Intermittent Fasting	Alternation of fasting periods (≤ 48 h) and ad libitum food intake	0 kcal alternating with ad libitum

Alternate day fasting	Total fasting or modified fasting on alternate days	0 kcal alternating with ad libitum
Time restricted eating	Periodic total fasting ≥ 14 h/day	No food intake during fasting, ad libitum during eating phase
Periodic fasting	Any type of fasting repeated at regular intervals	Depends on fasting method applied
Caloric restriction (CR)	undefined	~ 70% of normocaloric intake (avoiding malnutrition)
Fasting mimicking diet (FMD)	5 days of FMD with 1 to 6 cycles per year	800 – 1100 kcal

2. Fasting metabolism

Metabolic switch

Fasting induces a complex array of metabolic and immunological changes crucial for maintaining homeostasis and adapting to energy scarcity[81]. A substantial difference between fasting and starvation is the preservation of the body homeostasis[82]. Food deprivation and low glucose availability lead to the mobilization of energy from adipose tissue and protein stores[81,83]. Three main phases of the response to food deprivation have been described[82]. In the initial phase of fasting, the body uses up all the nutrients and energy provided then mainly relies on glycogen, lipids and protein stores to ensure the functioning of vital processes. The second phase is initiated once the glycogen stores have been completely exhausted and gluconeogenesis occurs, first from amino acids, which are then replaced by glycerol derived from adipose tissue as main provider for metabolic substrates, including ketone bodies[82]. The third and last phase is the total depletion of adipose tissues and a switch to using tissue proteins as endogenous energy source, which can be observed during famine or anorexia nervosa[82]. This phase is however not initiated during supervised and controlled prolonged or intermittent fasting interventions and is not subject of this thesis.

Under normal circumstances, ATP is mainly provided by glucose via glycolysis. The change from glucose to fat and ketone metabolism during fasting is called metabolic switch and starts 10-16h after the last food intake or string reduction of energy intake[70]. First a glucose drop is observed, followed by a decrease in insulin levels preceding the metabolic switch. The exact time of onset depends on the glycogen stores in the liver and the nutrient composition of the preceding meal, physical activity and overall energy expenditure depend on the basic metabolic rate. During periods of low glucose availability, the pancreas increases glucagon secretion and reduces insulin release. This hormonal shift results in decreased glucose storage. Glucagon binds to its receptors on the liver, triggering a cyclic AMP cascade that activates glucose phosphorylase. This enzyme catalyses the release of glucose-1-phosphate, which is subsequently converted to glucose-6-phosphate and hydrolysed to produce glucose[81]. Glycerol from FAs is converted to glycerol-3-phosphate and then to dihydroxyacetone, which

enters glycolysis[28]. FAs are transformed into fatty acyl CoA, entering β-oxidation for energy production[28]. The depletion of glycogen stores takes approximately 24 hours, and glucagon activates hormone-sensitive lipase, which, along with triglyceride lipase, breaks down triglycerides into free fatty acids (FFA) and glycerol. Part of these FFAs is transformed into ketone bodies by the liver and used as energy sources and signalling molecules. Ketone bodies like BHB, acetoacetate and acetone regulate the expression of several transcription factors such as PGC-1a, sirtuins, ADP-PARP1 and NAD+[70]. This metabolic switch can be detected by measuring BHB and pyruvate dehydrogenase kinase isoform 4 (*PDK4)* expression[84].

A decrease in the ATP/AMP ratio leads to the activation of AMPK and cytoprotective enzymes such as peroxidase, catalase and glutathione S-transferases. A plateau of ketosis is reached after approximately four days of fasting and the switched metabolism allows reduced protein utilisation regulated by mTOR signalling pathway inhibition. The usage of ketones as energy source during weight loss promotes retention of lean mass by decreasing overall protein synthesis and inducing autophagy leading to recycling of endogenous proteins[70]. Ketosis also leads to acidosis which has been hypothesised to explain the absence of hunger during extended fasting periods[70]. It has been reported that blood glucose remains stable, once settled at lower level, for the duration of the fast as long as fat reserves can provide fuel[70]. Low levels of IGF-1, together with the shutting down of nutrient-dependent signalling pathways such as mTOR and protein kinase A (PKA), lead to non- or low-dividing states of cells promoting their multi-stress resistance, increased DNA repair and a decrease in inflammation[70]. Fasting also leads to changes in the gut microbiome composition, a decrease in reactive oxidative stress, and impacts some important regulators such as SIRTs, mTOR, nuclear factor erythroid 2-related factor (Nrf2), FOXO1 and NF-κB. These metabolic implications of fasting will be explained in greater detail below[70].

Nrf2, sirtuins and AMPK energy homeostasis

As mentioned above, the low carbohydrate intake leads to a depletion of the glycogen stores and a subsequent mobilisation of FAs, which stimulate the hepatic ß-oxidation and ultimately lead to the production of ketone bodies, acetoacetate, which is reduced to ß-hydroxybutyrate, and acetone[85,86]. These ketones have been reported to activate several transcription factors amongst which figure cyclic AMP response element binding protein (CREB), NF-κB and brain derived neurotrophic factor (BDNF)[85]. An increased mitochondrial function has been observed after a rise in ROS and pro-inflammatory mechanisms induced by ketones. These pro-inflammatory and detrimental cell activities are, however, followed by an adapted stress response mediated by the upregulation of master regulators as nuclear Nrf2 reducing inflammation by decreasing NF-κB expression, sirtuins and AMP-activated kinase. In

addition, improved DNA repair and autophagy has been observed, following the rise in ROS production[86]. After an initial increase of ROS production, fasting also induces an NAD+/NADH decrease, activating sirtuins 1 and 3[86].

Sirtuins (SIRTs) are nicotine adenine dinucleotide(+)-dependent histone deacetylases regulating critical signaling pathways. These proteins belonging to the class III histone deacetylases include seven different enzymes named from 1-7[87]. An important activity of SIRT3 is the deacetylation and activation of NADP-dependent isocitrate dehydrogenase leading to an increased NADPH production, neutralizing lipid peroxides*[86].* BHB has been suggested to specifically induce SIRT3 and SIRTs have been reported to be increased after 24H of fasting and during IF[86,88,89]. This increase is observed to a larger extent during IF than during continuous CR[89]. In general, SIRTs play important roles in inflammation, metabolism, oxidative stress and apoptosis and interestingly a downregulated expression has been observed in adipose tissue from obese women and in peripheral blood mononuclear cells (PBMCs) from subjects with insulin resistance*[87,89].* Besides fasting and CR in general, also a decrease in BMI and specific diet derived polyphenols can lead to an activation of SIRTs*[89].*

A third sensor of energy homeostasis is the 5'-AMP-activated protein kinase (AMPK) located in the hepatocytes. A decrease in AMP/ATP ratio leads to an upregulation of AMPK, subsequently inducing downstream target peroxisome proliferator-activator receptor alpha (PPARa) which impairs the secretion of chemokine CCL2, leading to a reduced mobilization of monocytes[90]. A rise in AMPK also inhibits anabolic pathways and stimulates catabolic reactions such as autophagy, leading to an elimination of damaged proteins and organelles and an overall improved mitochondrial function, partially mediated via FOXO3a[85,86].

AMPK also inhibits the mechanistic target of rapamycin (mTOR) both via phosphorylation and activation respectively inhibition of the mTOR negative regulator tuberous sclerosis complex (TSC2) and the mTORC1 component regulatory-associated protein of mTOR (Raptor)[91]. These processes induce a subsequent decrease in protein synthesis and apoptosis[91]. Especially mTOR complex 1 (mTORC1) plays a key role in regulating energetically linked cell processes such as cell growth, protein, lipid, cholesterol and nucleotide synthesis while inhibiting cell autophagy[92]. Autophagy is a cellular mechanism implicated in cellular homeostasis by degrading aged or damaged organelles by secretion of lysosomes[93]. An upregulation of the mentioned processes has been associated with a prolonged life span[85]. Interestingly mTOR is still inhibited even in AMPK knock-out models, indicating other mTORC1 regulators such as Ras-related CTPases or leucyl-transfer RNA synthetase 1[91]. It has been reported that the failure of inactivating mTORC1 during glucose deprivation induces ATP depletion and subsequent cell death[91]. The cell protective activities promoted by AMPK upregulation largely overlap with the ones initiated by Nrf2 and SIRTs (Figure 6).

Thus, during ketosis, a stress situation inducing signaling via Nrf2, SIRTs and results in a cell protective response mediated by improved mitochondrial function, increased DNA repair and autophagy as well as by anti-oxidative, anti-inflammatory states and a reduced anabolic metabolism[86].

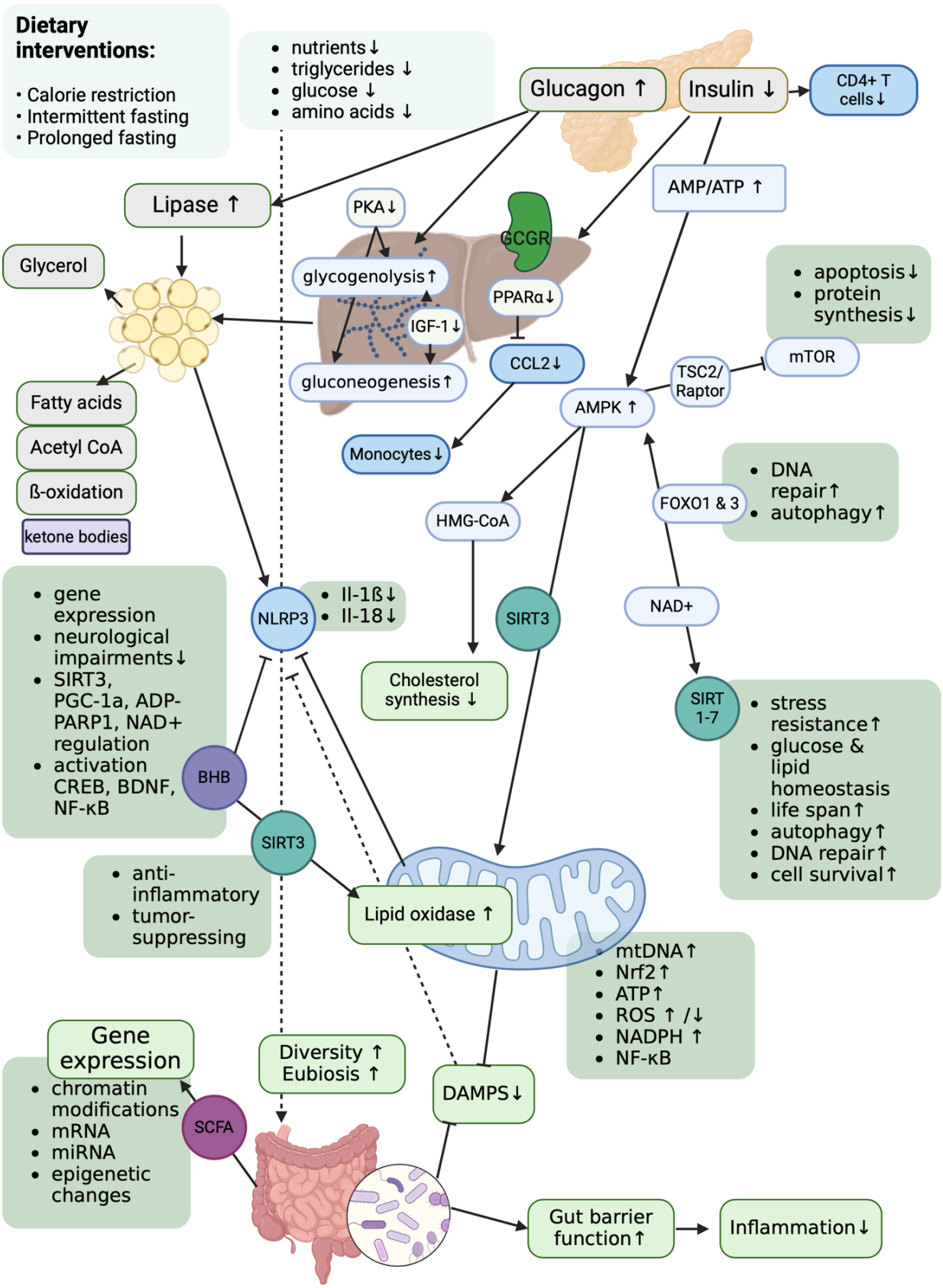

Figure 6: Fasting metabolism – proposed mechanisms.

The figure describes the complex underlying mechanisms that have been proposed to mediate the beneficial health effects of fasting. The initial dietary intervention induces a decrease in nutrients triggering the onset of the represented metabolic cascade. The green boxes show possible effects induced by the respective key players. AMP, adenosine monophosphate; AMPK, Adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; BDNF. Brain derived neurotrophic factor; BHB, Beta-hydroxybutyrate; CCL2, chemokine ligand 2; CREB, cAMP-response element binding protein; DNA, Deoxyribonucleic Acid; FOXO, Forkhead box protein; GCGR, glucagon receptor; HMG-CoA, Hydroxymethylglutaryl-CoA; IGF-1, insulin like growth factor 1; IL, interleukin; DAMPs, damage associated molecular patterns; mRNA, messenger ribonucleic acid; miRNA, micro messenger ribonucleic acid; mTOR, mammalian target of rapamycin; NAD+, Nicotinamide adenine dinucleotide; NADPH, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen; NF-kB, nuclear factor-kappa B; NLRP3, nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3; Nrf2, nuclear factor erythroid 2–related factor 2; PKA, protein kinase A; PGC-1a, Peroxisome proliferator-activated receptor-gamma coactivator; PPARa, peroxisome proliferator-activator receptor alpha; ROS, reactive oxygen species; SIRT, sirtuin; TSC2, Tuberous sclerosis complex 2. Created in biorender. Hansen B, 2024

2. Immunometabolism - Anti-inflammatory effects of fasting and underlying mechanisms

Inhibition of NLRP3 inflammasome

Nucleotide-binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome activity is a critical component of the innate immune system and one of the hallmarks of chronic inflammation[94,95]. Inflammasomes are defined as multi-protein signalling platforms consisting of a pattern recognition receptor (PRR) and an adaptor, mediating caspase-1 activation and the secretion of proinflammatory cytokines IL-1ß and IL-18[95]. IL-1ß is associated with fever, pain, vasodilatation and facilitating infiltration of immune cells into infected tissues, while IL-18 is involved in IFN-y secretion [95]. The PRR of the NLRP3 inflammasome can recognize several stimuli, including mtDNA acting as DAMPs, bacterial and fungal components, ionflux and lysosomal damage amongst others, while the role of ROS in NLRP3 activation remains controversial[95]. It has been reported that serum IL-1ß levels are lower in fasted than fed subjects[94]. Arachidonic acid, which is elevated during fasting, has been suggested to inhibit NLRP3 by decreasing phospholipase C activity to reduce JNK1 stimulation and therefore explaining the inverse relationship of AA and IL—1ß serum levels during fasting[94]. Another possible explanation for the inhibition of NLRP3 during fasting is a decrease in DAMPs released in the form of cardiolipin, ROS or mtDNA by mitochondria into the cytoplasm[96]. Also, ketone bodies, mostly BHB have been reported to suppress NLRP3 activation, most likely via acting on K+ efflux and preventing signaling ions to induce the formation of the NLRP3 complex, via improving oxidative stress resistance or reducing ER stress, however, the mechanisms are incompletely understood[97].

Lower CD4+ T cell expression via decreased insulin and IGF-1 levels

A decrease in insulin is an obvious consequence of fasting and is one of the most studied effects. The anabolic hormone secreted by ß-cells of the pancreas promotes carbon energy deposition in the body upon energy availability[98]. Hyperinsulinemia has several pathological effects such as increased tubular reabsorption of sodium an unfavourable effect on lipid metabolism[86]. An increase in insulin also leads to an upregulated synthesis of insulin-like growth factor 1(IGF-1), an anabolic hormone secreted from liver cells[99]. Fasting naturally leads to a decrease of both anabolic factors, who have been reported to be involved in apoptosis and cellular metabolism, aging and inflammation[99]. They also regulate over 50% of the differentially expressed genes, most of which are involved in lipid and cholesterol biosynthesis, but they also suppress the expression of genes involved in autophagy[100]. Additionally, both metabolic hormones increase CD4+ T cell expression via their respective receptor signaling, IGF-1 acts specifically on Th17 cells[99].

[101]

Anti-inflammatory effects of hunger mediated by AgRP circuits

Hunger has been previously reported to have a stronger anti-inflammatory effect than NSAIDs, indifferent of gender, age or body weight[101]. Agouti-related protein (AGRP) is a natural antagonist of the melanocyte stimulating hormone and its upregulation, which has been observed during fasting, leads to increased hunger[102]. AGRP has been suggested to mediate the anti-inflammatory effects of hunger via descendent vagal efferent signalling after activation of PVH neurons[101]. A hypothesis is that in times of food scarcity the neural circuits prioritize the functions that are most crucial for survival and inhibit long-term inflammatory pain[101].

Inflammation and fasting induced changes of the gut microbiome

As mentioned above, the gut microbiome plays a crucial role in several diseases, including RA. Its composition is strongly influenced by dietary intake and therefore by fasting. The abundance of the species *Christensenella*, assocaited with longevity, increased after fasting and inversely correlated with age as well as body mass index (BMI)[84]. Significant changes in the abundances of Proteobacteria and Fusobacteria were observed after PF, especially a decrease in *Euryarchaeota* and an increase in *Cyanobacteria*, as well as a decrease in the *Firmicutes/Bacteroidetes* ratio has been reported[84]. While PF has an important impact on the gut microbiome composition, IF might be even more relevant in the long-term gut health. Contrary to FMD or PF, an important aspect unique to TRE is the consistency of eating pattern and a harmonization and synchronisation with the circadian rhythm of natural metabolic pathways and the gut microbiome. This is of major importance as diurnal shifts in the gut microbiome composition have been observed and associated with host inflammation[54]. These beneficial shifts in

the gut microbiome composition induced by fasting might subsequently lead to an improved gut barrier, and, interestingly a disrupted gut barrier is a hallmark of mucosal inflammation, possible playing a key role in the mucosal origins hypothesis of RA[12,103].

3. Beneficial health effects of fasting

The numerous effects of PF and IF and their effect on inflammation lead to several health benefits (Figure 7). Fasting has been associated with enhanced psychological wellbeing, including decreased anxiety and depression, neuroprotective properties improving cognitive function, improved metabolic health, due to regulation of important cardiovascular health markers such as cholesterol, triglycerides and blood pressure, a decreased risk for type 2 diabetes (T2D), mediated via increased insulin sensitivity and weight loss, a reduced clinical disease activity in rheumatoid arthritis and an anti-cancer as well as a chemotherapy supporting effect in humans[104-107]. Some of the neuroprotective effects might be related to the involvement of ketone bodies in neurological processes, increasing adaptive responses and improve memory deficits and anxiety-like behaviours[108]. The cancer preventive and antitumour effect could be the high dependency of glucose of cancer cell growth, a decrease of IGF-1 during IF or also the increased autophagy in malignant cells induced via an AMPK activation and mTOR pathway inhibition by IF[109]. Another beneficial effect of IF is a reduction of visceral fat in pregnant women without negatively impacting foetal growth. Although there is lacking evidence to confirm IF as a safe practice in pregnant women, it offers a promising dietary intervention to prevent negative long-term health effects for the offspring of obese women[109].

Several clinical trials have shown efficacy and safety of IF in patients with T2D and metabolic syndrome, for other conditions however, more high quality studies are still needed to confirm these positive health associations of fasting in humans[110,111].

Thus, we can conclude that fasting may lead to overall health benefits, which can, however, usually not be sustained long-term in RA and symptoms reappear after food reintroduction[70]. Although it has been shown previously that the beneficial effects might be extended to up to one year by following a vegetarian or vegan diet, this has to be further studied, and the underlying mechanisms are not understood. Nonetheless, fasting, both IF and PF, are promising and valuable intervention strategies for disease prevention or treatment that must be further studied in detail.

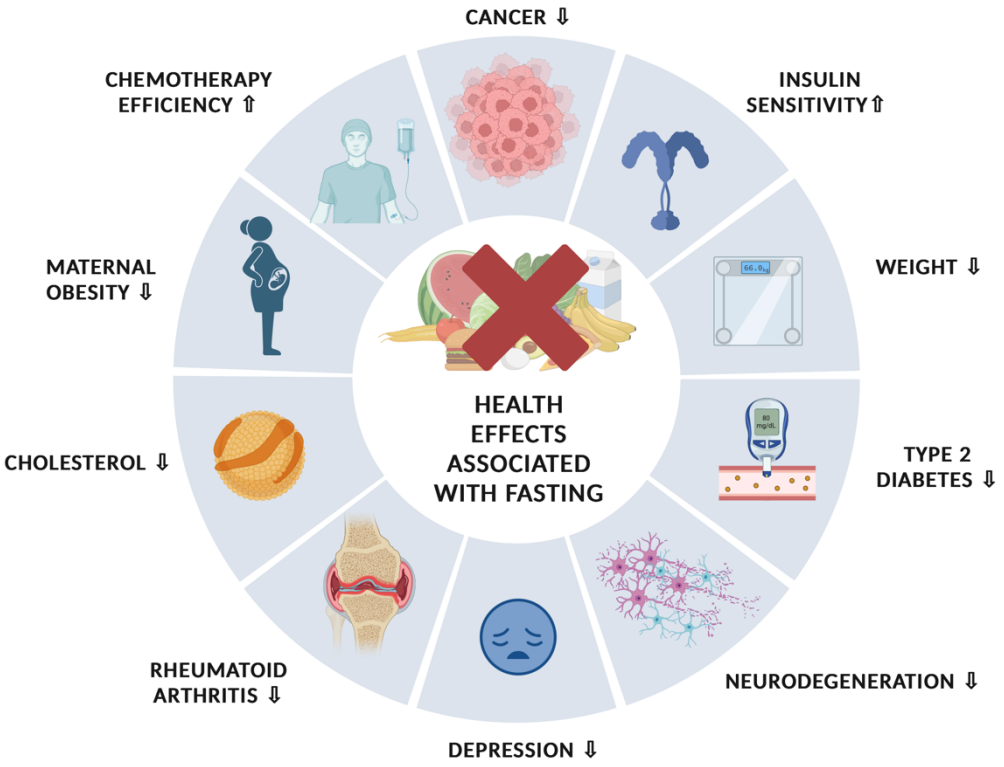

Figure 7: Health effects associated with fasting in humans.

This figure shows several beneficial health impacts that have been previously reported to be associated with fasting. Underlying mechanisms are, however, mostly unclear.

D. Anti-inflammatory diets

Chronic inflammation is a key player and initiator of several diseases, including T2D, cancer, atherosclerosis, asthma and depression[112]. Adapted lifestyle choices, such as the integration of anti-inflammatory nutrients, as well as implementation of specific food and dietary pattern, emerge as essential mediators to control inflammatory responses[113]. Some of the anti-inflammatory dietary pattern have been tested in relation to chronic diseases including an elimination diet, gluten-free diet, low-calorie, low-fat as well as low-sodium diets and also mediterranean, vegan and ketogenic diets[114]. A typical anti-inflammatory (AI) diet includes consumption of unrefined and minimally processed foods, including high amounts of fibre as well as MUFAs and PUFAs and lean protein sources, while reducing the consumption of red meat and highly saturated or trans-fat foods[113]. The AI diet has been reported to lead to weight loss, alterations in the gut microbiome, reduced depression risk and symptoms, improvements in fatigue and fertility, increased heat shock protein secretion and decreased symptoms in NCDs such as RA and non-alcoholic fatty liver disease [113,115-120].

As categorization tool of individuals' diets on a continuum from anti- to pro-inflammatory, the dietary inflammatory index (DII) was developed and has been updated according to recent literature, including

45 different food parameters[112]. One of the most prominent AI diets is the MD. It is the most well-known and best researched dietary pattern worldwide[121]. In 2010 UNESCO defined the MD as a "set of skills, knowledge, practices and traditions ranging from the landscape to the table, including the crops, harvesting, fishing, conservation, processing, preparation and, particularly, consumption of food"[52]. The diet favours a high intake of vegetables, whole grains, fruits, legumes, fish and olive-oil, a moderate intake of dairy, poultry and eggs and a very low consumption of red meats and refined sugars[122]. Extra-virgin olive oil, a source of essential dietary FA, vitamins, polyphenols, chlorophylls and phytosterols, is considered as a major reason for a long-life span amongst Mediterranean populations[121]. Its secoiridoids, including oleocanthal, oleacein, oleuropein and ligstroside, have been reported to have antioxidant, anti-inflammatory and anti-proliferative properties, acting thus anti-cancerous[123]. Additionally, estrogenic molecules like biochanin A, or coumestans can compete with endogenous oestrogens and potentially reduce their mitogenic activities[124]. Legumes are mainly known for their health effects mediated by fibre and flavanols, reducing blood pressure, cholesterol and endothelial dysfunction[121]. Dairy products are generally consumed in a fermented form such as yogurt or cheese[121]. Although many of these foods provide important phytonutrients with anti-inflammatory, antioxidant, neuroprotective, cardioprotective, anti-diabetic, antimicrobial and anti-steatosis effects, the impact of individual foods should not be overestimated[121,122]. In general a high adherence to a MD is associated with a reduction of pro-inflammatory cytokines, reduced oxidative stress and CRP levels as well as a decreased risk for several inflammatory diseases as CVDs, T2D, obesity and metabolic syndrome[52,122]. Some of these health benefits could be linked to a gut microbiome alteration with increased SCFA production and improved intestinal barrier integrity[122]. It has also been suggested that the decreased intake of the pro-inflammatory trimethylamine-N-oxide (TMAO), which main sources are red meat, eggs and dairy, is a key player in mediating beneficial effects on CVDs amongst others[122]. Also, a MD has been shown to alter certain pathways associated with increased cardiovascular risk by modifying gene expression[121]. Thus, high adherence to an AI diet, in particular MD, has shown numerous short and long-term benefits, induced by several underlying mechanisms which need to be further elucidated.

E. Nutrition and rheumatoid arthritis

Both the disease RA and the possible health effects of fasting and AI diets have been elaborated in detail above. Although the exact underlying causes and mechanisms of autoimmunity are not fully understood, there is a complex interplay of environmental factors including lifestyle, diet and intrinsic factors as genetics[52]. Sköldstam et al. studied the effect of fasting and a lactovegetarian diet on RA in 1979 and found significant improvements of RA disease activity, including reduced pain and stiffness after the fasting intervention[125]. In 2020 a meta-analysis on dietary interventions in RA was published by Philippou et al., encompassing fasting, supplementation, MD and other dietary interventions. A total of 70 studies was included with the majority focusingg on dietary supplementation[7,52]. Fasting or caloric restriction was only investigated in 5 studies, a ketogenic diet in 1 study and a MD diet in 4 studies, while other reported dietary patterns included a vegetarian, vegan or gluten free diet. Studies addressing fasting or CR in RA suggested an overall improvement in RA symptoms, including a reduction in morning stiffness and a decreased number of painful joints[52]. Particularly the MD, characterized by a high consumption of fruits, vegetables, whole grains, legumes, nuts and olive oil, as well as vegetarian, vegan and gluten free diets have shown promising benefits for individuals with RA[7]. The most common eliminated food items included dairy products, egg, meat, fish, refined sugars, wheat, corn, nuts, citrus fruits and coffee[126]. Sodium chloride has been suggested to have a negative impact on RA by activating proinflammatory macrophages, Th17 and decreasing Treg cells, while positive effects are associated to a higher consumption of omega-3 FA, vitamin D and several phytochemicals based on their ability to reduce pro-inflammatory cytokines[127]. Although fasting seems to have beneficial effects on both subjective and objective variables in RA, symptoms usually reappear after reintroducing food and therefore the intervention has been classified as unsustainable[7]. Some beneficial effects of fasting could, however, be sustained for one year by implementing a vegetarian diet[128]. Body composition has been linked to RA and white adipose tissue is considered a source of pro-inflammatory mediators fueling the disease[122]. Also, eicosanoids formed from arachidonic acids, derived mainly from animal based food sources, as mediators of inflammation are hypothesized to play a key role in RA[129].

Since the publication of the above mentioned systemic review in Nutrition Reviews in 2020, additional clinical trials focusing on diet in RA have been conducted, amongst others the NutriFast study performed by our collaborators at the Charité in Berlin with partial data analysis at the University of Luxembourg[1,130]. This clinical trial compared an anti-inflammatory diet recommended by the DGE with PF followed by a PB diet. Significant improvements were reported for both groups after a study period of 12 weeks[131]. Another clinical trial worth mentioning is the Plants for Joints randomized controlled trial by Walrabenstein et al., investigating the impact of whole-food plant-based diet in combination with physical activity and stress management in patients with RA. The intervention group showed

significantly greater improvements than the control groups after 16 weeks[132]. A special emphasis was put on critical nutrients of a plant-based diet, including protein, omega-3 FA, iron, zinc, iodine and calcium but the overall energy and macronutrient intake did not differ from the control group[132].

Overall, diet has garnered significant attention for its potential in influencing the onset and progression of RA[122]. The most promising hypotheses suggest underlying mechanisms based on the impact of diet on the gut microbiome composition, the interaction with the immune system and direct beneficial or detrimental effects of specific nutrients. IF has been previously reported to significantly impact the gut microbiome composition in other autoimmune diseases, such as multiple sclerosis, inducing enrichments of *Lactobacillaceae, Bacteroidaceae* and *Prevotellaceae* families, associated with decreased Th17 activity and enhanced Treg cell secretion, reducing overall inflammation[133]. Besides fasting interventions, also specific dietary compounds as fibre or polyphenols have been linked to beneficial changes in the gut microbiome composition[134,135].

This dietary connection to emerging research fields as the gut microbiome and its impact on NCDs such as RA are of high interest and need further research. Deciphering the changes happening during PF and TRE in patients with RA and analysing dietary patterns to sustain the beneficial effect of PF are crucial[1]. Different fasting mechanisms, a detailed study design and protocol, results of deep immunophenotyping as well as the outcome of one week of PF followed by TRE will be elaborated in chapter V.

V. Results

A. Manuscript I:

Perspective: The Impact of Fasting and Caloric Restriction on Neurodegenerative Diseases in Humans.

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1. Contribution

As the first author of this perspective, I was responsible for the conceptualisation of the research approach, the planning and drafting of the manuscript outline, the literature research and the writing process together with Kirsten Roomp. The figure was created by myself using the Biorender software.

2. Background and introduction

Fasting plays a major role in this thesis project and in the framing ExpoBiome study, particularly concerning its implications for chronic diseases characterized by localized and systemic inflammation, such as RA and PD. Overall, the importance of lifestyle factors, specifically diet, in the development and progression of NCDs is increasingly recognized. Prior research has reported beneficial outcomes from fasting interventions in RA, emphasizing the need for a comprehensive state-of-the-art review of fasting in the context of NDs. However, while conducting the literature research, it became evident that, in contrast to RA, no human trial looking at the impact of fasting interventions on PD has been previously conducted. Although several studies have explored the potential of other dietary interventions as a Mediterranean or a ketogenic diet, both showing beneficial impacts in patients with ND, fasting and caloric restriction (CR) remain underexplored. Recognizing this research gap, this perspective, aims at at highlighting the possible effects of the so far neglected area of fasting and CR in ND. As the literature on PD and fasting is very scarce, we decided to broaden the topic of our perspective on the impact of fasting on ND in general.

The perspective provides an extensive summary of primary intervention studies and explores the mechanisms through which these dietary interventions influence neuropathology. It emphasizes the urgent need for high-quality, longitudinal clinical trials to deepen our understanding of the immune-metabolic mechanisms involved. The article also examines metabolic transitions, such as the switch from glucose to ketone-based energy during intermittent fasting, which has been associated with potential health benefits.

The findings of this article could be particularly valuable to the clinical community, especially given the rapid aging of the population. Nutritional interventions could be crucial in managing and potentially decelerating the progression of neurodegenerative diseases, presenting a non-pharmacological strategy for disease management. The concept of metabolic flexibility has been demonstrated to reduce oxidative stress and inflammation, both of which are significant contributors to neurodegeneration. Additionally, fasting and caloric restriction may induce autophagy, potentially mitigating the accumulation of alpha-synuclein in PD or amyloid-beta in Alzheimer's disease. These mechanisms highlight the potential for dietary interventions to offer neuroprotective effects and improve overall brain health and are elucidated in greater detail in the manuscript.

Our perspective synthesizes existing knowledge and identifies gaps in the current research landscape, emphasizing the need for robust clinical trials. Such studies would not only explore the efficacy of fasting and caloric restriction in ND but also elucidate the underlying biological mechanisms. By doing so, future research, as the ongoing ExpoBiome study, could pave the way for new dietary guidelines

and therapeutic strategies that leverage metabolic interventions to combat neurodegenerative diseases.

3. Manuscript

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Perspective

Perspective: The Impact of Fasting and Caloric Restriction on Neurodegenerative Diseases in Humans

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A B S T R A C T

Neurodegenerative diseases (NDs) are characterized by the progressive functional and structural denaturation of neurons in the central and peripheral nervous systems. Despite the wide range of genetic predispositions, the increased emergence of these disorders has been associated with a variety of modifiable risk factors, including lifestyle factors. Diet has been shown to influence cognitive alterations in the elderly population with age-related brain pathologies, and specific dietary interventions might, therefore, confer preservatory protection to neural structures. Although Mediterranean and ketogenic diets have been studied, no clear guidelines have been implemented for the prevention or treatment of ND in clinical practice. Murine models have shown that intermittent fasting and caloric restriction (CR) can counteract disease processes in various age-related disorders, including NDs. The objective of this perspective is to provide a comprehensive, comparative overview of the available primary intervention studies on fasting and CR in humans with ND and to elucidate possible links between the mechanisms underlying the effects of fasting, CR, and the neuropathology of ND. We also included all currently available studies in older adults (with and without mild cognitive impairment) in which the primary endpoint was cognitive function to provide further insights into the feasibility and outcomes of such interventions. Overall, we conclude that nutritional intervention trials focusing on fasting and CR in humans with ND have been neglected, and more high-quality studies, including longitudinal clinical intervention trials, are urgently needed to elucidate the underlying immune–metabolic mechanisms in diet and ND.

Keywords: neurodegenerative disease, fasting, caloric restriction, ketogenic diet, Alzheimer's disease, Parkinson's disease, multiple sclerosis, mild cognitive impairment, elderly, human

Statement of Significance

This perspective provides a pioneering synthesis of clinical intervention trials examining the effects of fasting and caloric restriction on individuals suffering from neurodegenerative diseases, marking a comprehensive analysis on this topic.

Introduction

Fasting (i.e., caloric restriction [CR] in various forms) has been used as an intervention to promote health since the beginning of civilization and has spread independently among different regions, cultures, and religions worldwide [1]. It is believed to have already been established as a treatment method by Hippocrates in the 5th century BCE and has been used ever

Abbreviations: AD, Alzheimer's disease; ADF, alternate day fasting; CR, caloric restriction; FA, fatty acid; FMD, fasting-mimicking diet; IF, intermittent fasting; KD, ketogenic diet; MCI, mild cognitive impairment; MD, Mediterranean diet; MDS-UPDRS, Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale; MS, multiple sclerosis; ND, neurodegenerative disease; PF, prolonged fasting; PD, Parkinson's disease; sNfL, serum neurofilament light chain; TRE, time-restricted eating; VLC, very low-carbohydrate.

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since by numerous medical schools to treat acute and chronic diseases [2]. Various practices of CR using fasting have repeatedly shown remarkable health benefits [3,4].

Neurodegenerative diseases (NDs) comprise a range of complex medical conditions that affect neurons in the brain and possibly extend to the spinal cord and the peripheral nervous system [5]. The most common such diseases are Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) [6]. NDs, especially AD, have been on the rise in recent decades, mostly because of aging populations [7]. Although recent studies on several different treatment approaches, such as monoclonal antibodies against plaques, stem cell therapy, or nanotherapeutics, present promising treatment approaches, no cure for these diseases currently exists [7–10].

Genetic factors and age are key players in the onset and development of NDs; however, environmental and lifestyle factors also play an important role in their development [11]. In addition to physical activity and cognitive exercise, nutrition has emerged as a major factor influencing ND pathology [12]. The Mediterranean diet (MD) and ketogenic diet (KD) have been associated with neuroprotective effects in ND, based on the inhibition of glycolysis, improved mitochondrial respiration, decreased production of reactive oxygen species, and prevention of neuronal apoptosis [13].

Although the beneficial effects of fasting have been observed in a wide variety of diseases, such as rheumatoid arthritis, or even during chemotherapy, a possible association with preventing or treating ND is still unclear [14,15]. Although a large body of work exists in animal models, including models for AD, PD, and stroke, showing that intermittent fasting and CR have beneficial effects on health and can counteract disease processes, few human studies have been conducted to date [16].

Here, we provide a comprehensive, comparative overview of the available data from primary intervention studies on fasting in humans with NDs, including all currently available studies in older adults (with and without mild cognitive impairment [MCI]) in which the primary endpoint was cognitive function, to provide further insights into the feasibility and outcomes of such interventions. Furthermore, we elucidate the possible links between the mechanisms underlying the effects of fasting and the neuropathology of ND (Figure 1).

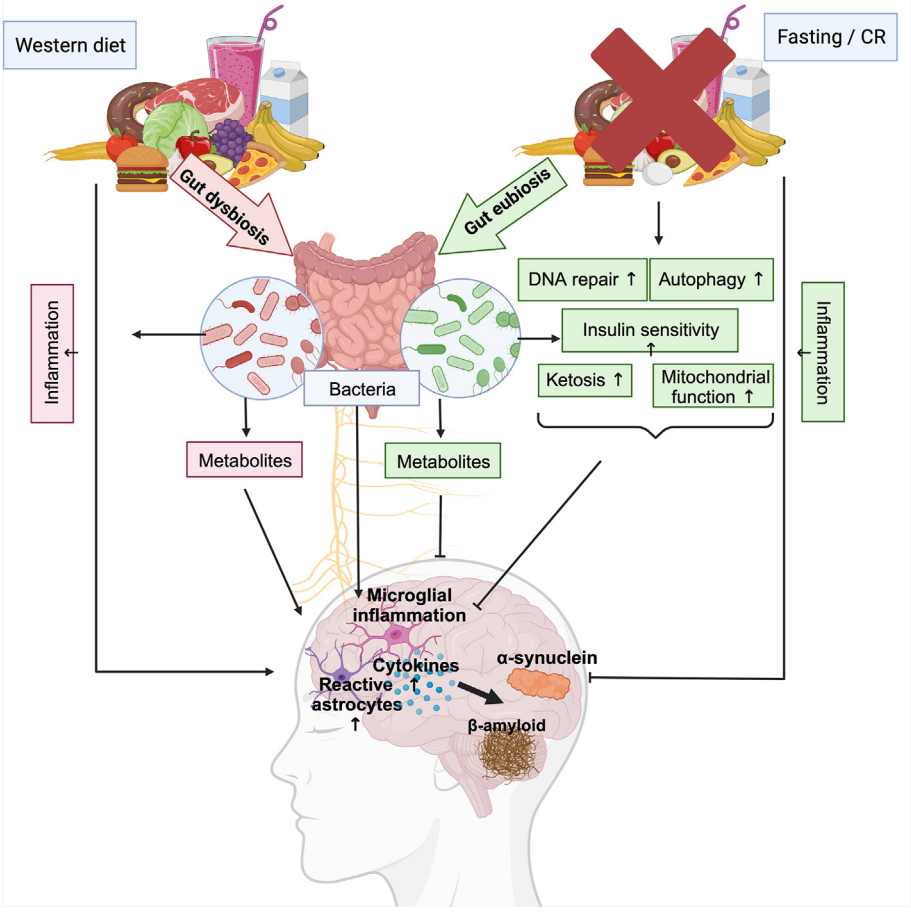

FIGURE 1. Underlying mechanisms. This figure illustrates the possible underlying mechanisms of the beneficial impact of fasting and CR on ND. While a typical western diet usually leads to gut dysbiosis and an increase in inflammation, the abstinence or reduction of food can have a positive impact on cognitive function by changing the gut microbiota composition and its metabolite secretion [41,42,46]. Several additional mechanisms including DNA repair, increased autophagy, upregulated mitochondrial function, increased insulin sensitivity, increased ketone body production, and decreased overall inflammation may result in beneficial impacts on cognitive function [73–75]. Abbreviations: CR, calorie restriction; ND, neurodegenerative disease. This image was generated using BioRender software (www.biorender.com).

Current Status of Knowledge

Fasting and caloric restriction

Fasting is defined as the voluntary abstinence from caloric ingestion for a limited time period. There are various forms of fasting and CR. Prolonged fasting (PF) typically lasts between 48 h and 21 d, with an intake of less than 350 kcal/d [17]. Intermittent fasting (IF) is an umbrella term that covers different approaches, including alternate day fasting, where complete or severe energy restriction occurs every other day, and time-restricted eating (TRE), where food intake is restricted to a specific time period each day. The most popular forms of IF are the TRE 16:8 method, with food intake ad libitum for 8 h followed by 16 h of fasting, and the 5:2 diet where calorie restriction (~600 kcal) occurs on 2 consecutive or nonconsecutive days per week. The latter is sometimes referred to as periodic fasting. CR is defined as a reduction in energy intake by at least 20% to 30% for 3 mo or more with an appropriate nutritional composition [18]. Finally, the fasting-mimicking diet (FMD), a combination of CR and IF, is becoming more popular. FMD is a low-calorie, low-protein, high unsaturated fatty acid (FA) diet characterized by periodic cycles of 3 to 7 d on a very low-calorie diet (~800–1100 kcal), providing essential macro- and micronutrients [19,20].

Alzheimer's disease

AD is the most prevalent ND and most common cause of dementia. More than 50 million patients are affected worldwide, and the numbers have increased dramatically over the past decades due to demographic changes in the global population, with people aged 65 and above outnumbering children under the age of 5 for the first time in 2018 [21,22]. AD is characterized by cognitive dysfunction, memory loss, and abnormal personality [22]. β-amyloid protein, the main component of senile plaques, and neurofibrillary tangles, composed of hyperphosphorylated tau protein, are considered key factors in the propagation of AD pathology [23]. Mendelian randomization studies provide evidence of a causal association between glycemic traits and AD [24]. Impaired glucose metabolism may be intrinsic to AD pathogenesis, contributing to oxidative damage, inflammation, and reduced energy metabolism [25].

A multitude of studies of dietary interventions and supplements has been conducted in humans with AD [26]. Medium chain triglyceride supplementation has shown promising effects in glucose hypometabolism in AD by increasing ketone levels and providing an alternative energy source to the brain [27,28].

However, to date, only one fasting study has been conducted on humans with mild AD or MCI. This randomized, placebo-controlled, 1-y study is ongoing and compares monthly, 5-d FMD cycles to a placebo diet where one meal is replaced with a pasta or rice-based meal 5 d per month. Twenty-eight patients have been enrolled to date (range 55–80 y), with the aim of enrolling 40 patients. The FMD group took a variety of dietary supplements for 25 d between the FMD cycles noted for fasting-mimicking and neuroprotective properties, whereas the placebo group did not. Initial data, 6 mo into the study, reported that 5 patients dropped out of each group for a variety of reasons, including poor acceptance and worsening nutritional status. FMD-emergent adverse events were mild to moderate. Diet compliance was good, and the authors considered 5-d FMD cycles administered once per month feasible and safe for patients with early AD or MCI. Information regarding potential cognitive changes will be reported at the end of the study [20].

Mild cognitive impairment

MCI is a transitional stage between the expected cognitive decline that occurs with age and the more serious decline observed in AD. Estimates of the etiology of AD dementia among patients with MCI range from 40% to 75% in different populations. The estimates include both those where MCI diagnosis was made clinically and in combination with biomarkers [29]. One interventional fasting trial and one CR trial have been conducted on humans with MCI.

Horie et al. [30] reported on a single-center, prospective controlled trial in obese patients suffering from MCI, aged 60 y or older. Patients were randomly allocated to conventional medical care alone ($n = 40$) or together with nutritional counseling ($n = 40$), aiming to promote weight loss through CR (a recommended calorie deficit of approximately 500 kcal/d for 12 mo). Significant weight loss was observed in all 75 patients completing the study, and cognitive test results improved without a difference between the groups. In the analysis, a decrease in BMI was associated with improvements in cognitive tests. Thus, the authors concluded that intentional weight loss was associated with improved cognition in MCI patients [30].

Ooi et al. [31] prospectively studied 99 elderly subjects with MCI of Malay ethnicity for 36 mo. The participants were grouped according to whether they regularly practiced IF (r-IF, $n = 37$), irregularly practiced IF (i-IF, $n = 35$), or nonfasters (n-IF, $n = 27$). IF was practiced by fasting on Mondays and Thursdays every week (Sunnah fasting) beginning from sunrise to sunset. Drinking was not permitted during fasting. After 36 mo, more MCI subjects in the r-IF group reverted to no cognitive impairment and lack of disease (categorized as successful aging) (24.3%) than those in the i-IF (14.2%) and n-IF groups (3.7%). The r-IF group showed a significant increase in the oxidative stress markers superoxide dismutase and malondialdehyde, activity and reduction in body weight, levels of insulin, fasting blood glucose, the inflammatory marker C-reactive protein, and DNA damage. Furthermore, metabolomics analysis showed that IF may modulate cognitive function via various metabolite pathways [31].

Parkinson's disease

PD is an age-related ND that affects 0.4% to 2% of the population over 65 y worldwide and is the second most common progressive neurodegenerative disease, with men being 1.5 times more likely to be affected than women [32,33]. Cardinal symptoms include motor deficiencies, such as tremors, bradykinesia, and rigidity, but also include a wide range of nonmotor symptoms, such as hyposmia, depression, insomnia, or cognitive impairment, constipation, and rapid eye movement sleep behavior disorders, severely impacting patients' quality of life [33–36]. The main neuropathological manifestations include neuroinflammation, degeneration of dopaminergic neurons, and accumulation of α-synuclein, a major component of Lewy bodies, in the dopaminergic substantia nigra [37,38]. The loss of dopaminergic neurons in PD involves mechanisms of inflammatory and autoimmune responses, with microglial activity being the major driver [39].

It is well established in the PD community that diet has a major impact on the disease. The 2 different dietary approaches shown to have beneficial effects on the outcome of PD are the MD and KD [40]. This is surprising, as these diets vary significantly in their composition. Whereas the MD is rich in antioxidants and fibers from fruits and vegetables, nuts, white grains, and healthy fats, the KD is usually high in saturated fats of animal origin and low in carbohydrates and fibers. These opposing dietary patterns imply the action of complex underlying mechanisms beyond the simple macro- and micronutrient composition. As both a high fiber intake and a metabolic switch have major impacts on the gut microbiome, the microbiota–gut–brain axis could be a key factor in modulating the onset and disease course of PD [41,42]. As fasting is also known to have a major impact on the composition of the gut microbiome, previous findings may indicate a beneficial effect of PF and/or IF on PD. However, no primary human studies have investigated fasting or CR in PD patients to date. A currently ongoing clinical trial, the ExpoBiome study, is investigating PF for the first time in patients with PD [43].

Multiple sclerosis

MS is a disease of the central nervous system, and while it is generally characterized as an autoimmune disorder, it is characterized by demyelination and neurodegeneration mediated by both T and B cells. MS is considered the leading cause of nontraumatic neurological disability in young adults. It is a heterogeneous disease in which most patients suffer from a relapsing form where discrete episodes of illness are followed by possible complete or partial remission, but 10% experience progression from the outset [44]. Metabolic syndrome and other closely related disorders such as diabetes and hyperlipidemia are overrepresented in patients with MS and are strongly associated with adverse outcomes [45]. Therefore, it is possible that CR and fasting may be important interventions impacting the development of disease [46].

Choi et al. [47] studied the effects of a low-calorie, low-protein FMD on patients with relapsing–remitting MS. A total of 60 patients were randomly assigned to a control diet ($n = 20$), the KD for 6 mo ($n = 20$), or a single cycle of modified human FMD for 7 d ($n = 20$) followed by the MD for 6 mo. Health-related quality of life and mental health were assessed at baseline, month 3, and month 6, and both the KD and FMD cohorts displayed meaningful to statistically significant improvements in all areas. The interventions were well-tolerated, and there were high compliance rates. However, adverse events were reported in all 3 groups, with airway infections (adverse events) and lower urinary tract infections (serious adverse events) being the most common [47].

In a more recent but smaller study, Fitzgerald et al. [48] randomly allocated 36 patients with MS to 3 diets for 8 wk: daily CR diet (22% daily reduction in energy needs), intermittent CR diet (75% reduction in energy needs, 2 d/wk; 0% reduction, 5 d/wk), or a weight-stable diet (0% reduction in energy needs, 7 d/wk). Adherence to daily CR was better than that to intermittent CR, with 86% completing the trial overall. Both CR diets were associated with significant improvements in emotional well-being and depression scores compared with the control weight-stable diet. No significant adverse effects were observed [48].

Bock et al. [49] studied a cohort of 60 relapsing–remitting MS subjects who were randomly allocated to a control diet ($n = 9$), a calorie restricted diet (single cycle of 7-d CR with 200–350 kcal/d was performed at study outset; afterwards a 3-d stepwise reintroduction to an isocaloric common diet, $n = 14$) or an adapted KD (average daily intake of <50 g carbohydrates, >160 g fat, and ≤ 100 g protein intake per day for 6 mo, $n = 17$). Serum neurofilament light chain (sNfL) measurements were performed at baseline, 3 mo, and 6 mo. sNfL levels are emerging biomarkers for neuroaxonal damage, and elevated sNfL levels are indicative of axonal injury [50]. An unexplained statistically significant increase in sNfL occurred at 3 mo in all 3 groups in an intragroup comparison. Only participants consuming the adapted KD showed a statistically significant decrease from baseline to 6 mo compared to the control group at the same time point.

The most recent study examined 10 relapsing–remitting MS patients that were randomized to an intermittent calorie restriction ($n = 5$) or control group ($n = 5$) for 12 wk. IF was defined as a reduction in daily calorie intake to ~25% of the usual intake on 2 nonconsecutive days per week. Significant improvements were observed in cortical volume and thickness, and neuroinflammation was mitigated [51].

Fasting and cognition in older adults

With age, many biological changes contribute to a progressive decline in physical function and cognition. Several contributing factors appear to accelerate this process, including low activity levels, excessive calorie intake, and body fat. Numerous studies in humans and animal models have shown that fasting has both beneficial and negative effects on cognition. Most human studies, however, have been performed in younger adults, with only a small number having been conducted in older adults.

In the oldest study by Witte et al. [52], conducted in 2009, 50 healthy, normal to overweight elderly subjects (mean age 60.5 y) were stratified into 3 groups: CR (30% reduction, $n = 20$), relative increased intake of unsaturated FAs (20% increase, unchanged total fat, $n = 20$), and control ($n = 10$). Memory performance was assessed under standardized conditions at baseline and after 3 mo of intervention. A significant increase in verbal memory scores after CR was observed, which was most pronounced in those with the best adherence to the diet. No significant memory changes were observed in the other groups [52].

Siervo et al. [53] recruited both middle-aged and older obese individuals. In the older age group ($n = 26$; mean age = 64.5 y), 12 individuals completed the study. Energy intake was reduced by 40% relative to an individual's calculated resting energy expenditure, and the weight loss target for each subject was 8% to 12% of the initial body weight. The duration to achieve this weight loss was 116.6 ± 27 d. Global cognitive performance, as measured by the Mini-Mental State Examination, only improved significantly in older individuals, whereas both age groups showed a significant improvement in the Trail-Making Test B, which measures visual search, scanning, processing speed, mental flexibility, and executive functions.

TRE was evaluated in a small group of 10 overweight adults (≥ 65 y) at risk for or with mobility impairment. The intervention was a TRE dietary pattern following the 16:8 method, which lasted for 4 wk. While compliance was high and significant mean weight loss was observed, no change in cognitive function was

measured using the Montreal Cognitive Assessment 30-point questionnaire for MCI. Few adverse events were reported (e.g., headache and dizziness) [54].

Hugenschmidt et al. [55] studied sedentary, obese adults (65–79 y) with normal cognition in a randomized trial comparing 3 groups: 20-wk aerobic exercise program only (n = 28), moderate (~250 kcal) CR with the exercise program (n = 30), or high (~600 kcal) CR with the exercise program (n = 30). The participants were evaluated at multiple time points for cognitive outcomes using a cognitive assessment battery. Randomization to CR did not significantly alter overall cognitive function compared to aerobic exercise alone, nor were there between-group differences in any individual executive function test up to 24 mo postrandomization. Compliance and adherence were excellent, and none of the participants dropped out because of an intervention-related adverse event.

A relatively large group of 107 elderly, obese individuals was randomly allocated to 4 groups: CR (500–750 kcal/d less than daily requirement, n = 26), CR plus exercise (n = 28), exercise only (n = 26), or control (n = 27). The goal was to achieve 10% weight loss in the first 6 mo, followed by weight maintenance for another 6 mo. Compliance was >82% in all 4 groups, and no adverse effects were noted. In the overall sample, cognitive function improved, but randomization to CR did not significantly change executive function compared to exercise alone. Furthermore, there were no between-group differences in any individual executive function test following the intervention or at long-term follow-up. Adding CR to exercise was associated with a modest improvement in the Mini-Mental State Examination score [56].

A small group of 11 sedentary, overweight, or obese older women (63–80 y) was randomly allocated to a 48-h zero-calorie diet with water provided ad libitum or their usual diet. A paired crossover design was used, with the interventions being at least 2 wk apart. Before-diet measurements were taken 1 d before the intervention, and after-diet measurements were taken immediately after the acute fasting ended. Cognitive performance was assessed using a test battery. The zero-calorie diet significantly prolonged the reaction time in a 2-choice reaction time test. Other cognitive tests were unaffected [57].

The largest study we identified included 185 obese, elderly individuals who were randomized to the MD plus CR lifestyle intervention (~25% CR to achieve ~5–7% weight loss, n = 75), MD lifestyle intervention only (n = 73), or their usual diet (n = 37). Participants were followed up for 14 mo, with the main measurements presented in the paper taken at baseline and 8 mo (completion of the active intervention phase). Although the CR group lost significant weight, the MD lifestyle intervention with and without CR did not significantly affect cognitive function compared with controls [58].

Parallels of fasting and ketogenic diet

To better understand the mechanisms of fasting and CR in humans with ND, it is worthwhile to examine several interventional KD studies conducted in patients with PD and AD. Both the effects of fasting and KD are thought to at least partly be based on the switch from glucose to fat metabolism and on a consequent change in the gut microbiome composition. KD is a low-carbohydrate, high-fat diet that induces a state of ketosis. Possible neuroprotective effects of KD through enhanced

TABLE 1
Interventional studies in individuals with NDs or the elderly (with cognitive function as endpoint)

Reference	Disease or condition	Main endpoint(s)	Diet(s)	Total n	Age (y)	% Female
Anton (2019) [54]	Elderly, obese	Cognitive function	TRE 16:8	10	M = 77.1	60
Bock (2022) [49]	Multiple sclerosis	sNfL levels	Fasting, KD, or control	60	M = 43.1, 45.7, 50.0	67, 76, 86
Choi (2016) [47]	Multiple sclerosis	Quality of life, mental health	FMD, KD, or control	60	M = 44.8	79
Fitzgerald (2018) [48]	Multiple sclerosis	Change in emotional well-being, depression	Intermittent CR, daily CR, or control	36	M = 37.4	81
Horie (2016) [30]	MCI, overweight	Cognitive function	CR or control	80	M = 68.1	83.7
Hugenschmidt (2019) [55]	Elderly, obese	Cognitive function	High CR plus exercise, medium CR plus exercise, or exercise only	88	M = 69.0	68
Napoli (2014) [56]	Elderly, obese	Cognitive function, quality of life	CR, CR plus exercise, exercise only, or control	107	M = 70, 70, 70, 69	65, 57, 61, 67
Ooi (2020) [31]	MCI	Cognitive function	Regular IF, irregular IF, or control	99	M = 68.7, 67.9, 69.1	37.8, 57.1, 44.4
Rahmani (2023) [51]	Multiple sclerosis	Cortical thickness, volume, perfusion, neuroinflammation	CR or control	10	M = 46	80
Rangan (2022) [20] [1]	AD or MCI	[Cognitive function][1]	FMD or control	28[1]	M = 71[1]	46[1]
Siervo (2012) [53]	Elderly, obese	Cognitive function	CR	26	M = 64.5	88
Solianik (2020) [57]	Elderly, overweight to obese	Cognitive function	PF, paired crossover design	11	Range = 63–80	100
Tussing-Humphreys (2022) [58]	Elderly, obese	Cognitive function	CR with MD, MD, or control	185	M = 66.3	85.9
Witte (2009) [52]	Elderly, normal to overweight	Cognitive function	CR, unsaturated FA enhancement, or control	50	M = 60.5	58

Abbreviations: AD, Alzheimer's disease; CR, caloric restriction; FMD, fasting-mimicking diet; IF, intermittent fasting; KD, ketogenic diet; MCI, mild cognitive impairment; MD, Mediterranean diet; ND, neurodegenerative disease; PF, prolonged fasting; sNfL, serum neurofilament light chain; TRE, time-restricted eating.

[1] Study recruitment is ongoing.

mitochondrial function, reduced inflammation, improved energy metabolism, and increased production of ketone bodies have been suggested [59].

The first trial of KD in patients with PD was published in 2005 by Vanitallie et al. [60]. Although improvements in Unified Parkinson's Disease Rating Scale scores could be seen, one must note that the sample size was small ($n = 5$) and the duration of diet implementation was only 28 d [60]. In 2021, Krikorian et al. [61] compared high-carbohydrate and very low-carbohydrate (VLC) diets in 23 older adults with MCI; after the 6-wk intervention period, significant cognitive improvement was observed in the VLC group. A significant improvement in the Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS) scores was observed in both arms of an 8-wk interventional trial by Phillips et al. [62], using low-fat diet compared to isolcaloric KD in 47 patients; the ketogenic group showed a significantly higher improvement for MDS-UPDRS Part 1 with a total reduction of 41% from baseline scores [62]. Koyuncu et al. [63] found a significant improvement in voice quality in patients with PD after consuming a KD for 3 mo.

Few studies have been conducted on KD in AD. Brandt et al. [64] found a significant cognitive improvement after using a modified Atkins diet for 6 wk. However, of the initially 27 enrolled patients, only 9 diet patients and 5 control patients completed the study [64]. The trial of Phillips et al. [62] reported in 2021 on the KD in patients with AD noted better daily function and quality of life, likely due to increased synaptic plasticity and reduced inflammation [52].

Ketogenesis creates ketone bodies like acetoacetate and β-hydroxybutyrate, serving as energy sources and offering antioxidative benefits that support mitochondrial function and reduce inflammation in Alzheimer's (65-67). Additionally, the microbiota–gut–brain axis is increasingly recognized in NDs where gut health has been linked to PD development [68]. Diet influences gut health significantly [33]. However, sustaining KD poses risks like malnutrition cardiovascular diseases, particularly in the elderly [69].

Conclusion

Of the 14 studies reviewed for this comprehensive perspective, 4 studies included patients with MS and 1 study included patients with AD. No fasting or CR studies were identified for PD patients, except for the ongoing ExpoBiome study [43], and all further studies included subjects with MCI or elderly normal weight, overweight, or obese subjects (Table 1). Studies in subjects with ND or MCI generally show positive effects on disease symptoms and/or cognition. However, the impact of fasting or CR on cognition in older adults produced heterogeneous results. The overall number of studies in humans is low. Other limitations include small sample sizes, short duration of the intervention, timing of cognitive measurements, or complex study designs that included exercise. Several studies focused on weight loss as the primary outcome, which can have a beneficial impact on cognitive function; however, the magnitude of weight loss did not correlate with the magnitude of cognitive improvement [70, 71].

Possible underlying mechanisms based on the metabolic switch have been elucidated in the section *Parallels of fasting and ketogenic diet*, as well as the risks and difficulties of the latter. PF or TRE can bypass the challenges encountered in a KD, as fasting is temporary, and a balanced diet can be reinitiated after the respective fasting periods. In addition, PF and TRE have been associated with positive outcomes in several conditions and diseases such as obesity, type 2 diabetes, and rheumatoid arthritis [14,72]. Patients with ND following a TRE pattern or PF could experience benefits beyond cognitive improvement [73–75].

Dietary recommendations for ND and the imminent application of the latter as a standard therapeutic intervention in daily clinical practice are of critical importance. Ongoing clinical trials, such as the ExpoBiome study [43], will provide insight into the mechanisms of fasting and the microbiota–gut–brain axis in relation to ND. Overall, this perspective emphasizes the need for additional clinical trials studying various fasting protocols, as this might potentially constitute a powerful new tool for preventing and treating ND.

Author contributions

The authors' responsibilities were as follows—BH, KR, JGS: conceptualized the research approach, planned and drafted the manuscript outline; BH, KR: wrote the paper; HE: contributed to literature research; JGS: reviewed and edited the manuscript; and all authors: read and approved the final manuscript.

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Conflict of interest

The authors report no conflicts of interest.

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4. Discussion

The results of the above perspective show that despite the compelling evidence from animal studies on beneficial effects of fasting and caloric restriction in health, there is a notable lack of clinical research exploring these dietary interventions in the context of non-communicable diseases (NCDs). This gap is evident not only in the study of NDs but also extends to other prevalent NCDs such as RA. Although some literature exists and several clinical studies have been conducted, particularly in prevalent NCDs as diabetes, robust long-term trials in humans remain notably scarce. This approach is reflected in the societal tendencies to prioritize treating established diseases over implementing proactive prevention strategies to mitigate their onset. This attitude highlights the urgent need for a mindset shift among patients, healthcare practitioners and researchers. Thus, the potential therapeutic benefits of nutritional strategies, including specific fasting protocols and caloric restriction, have not been sufficiently investigated in clinical settings. As a result, the promising findings from preclinical studies have not been translated into practical, evidence-based guidelines for patients suffering from these diseases. It highlights the need for well-designed clinical trials to explore the role of nutrition in managing NCDs, potentially leading to novel, non-pharmacological approaches to treatment and prevention. Expanding research in this area could significantly enhance our understanding and offer new hope for those affected by these debilitating conditions.

B. Manuscript II:

The impact of Fasting and Caloric restriction on Rheumatoid Arthritis in Humans: A narrative review.

Bérénice Hansen, Marta Sanchez-Castro, Lynn Schintgen, Arefeh Khakdan, Jochen G. Schneider, Paul Wilmes, *Clinical Nutrition, 2025*

1. Contribution

As the first author of this perspective, I was responsible for the conceptualisation of the research approach, the planning and drafting of the manuscript outline, the literature research and the writing process together with Marta Sanchez-Castro. The figures were created by myself using the Biorender software.

2. Background and introduction

As already elaborated for the first manuscript, fasting and nutrition are of major importance in this thesis project and in the framing ExpoBiome study, especially regarding RA and PD. This narrative review was written as during the thesis project the sparsity of available literature on RA and fasting was noticed and a comprehensive of the overall data is considered as important.

This narrative review summarises all the outcomes of primary intervention studies of fasting in patients with RA in a similar matter as the manuscript I does for NDs.

The findings of this article offer a reliable and valuable source to the clinical and science community on previously reported scientific findings in RA. The narrative review highlights the lack of understanding of fasting mechanisms in auto-immune diseases as well as the promising possibilities dietary interventions can offer.

3. Manuscript

Clinical Nutrition

The impact of Fasting and Caloric restriction on Rheumatoid Arthritis in Humans: A narrative review.

--Manuscript Draft--

Manuscript Number:	YCLNU-D-25-00297
Article Type:	Narrative Review Article
Keywords:	Rheumatoid arthritis; Fasting; Time-restricted eating; Microbiome; Caloric restriction; Inflammation
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	Paul Wilmes
Abstract:	Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease affecting approximately 1% of the global population. It is characterized by swollen and painful joints eventually evolving into bone erosion and cartilage degradation and systemic inflammation, significantly reducing patients' quality of life. While modern pharmacological treatments often lead to symptom improvement, they are accompanied by substantial side effects, which can further impair patient wellbeing. Dietary interventions, particularly fasting and caloric restriction (CR), have gained increasing attention as adjunctive strategies for RA prevention and treatment. Their anti-inflammatory potential and ability to modulate the gut microbiome render them an attractive option to accompany or modify medical treatment. However, high-quality research on fasting and CR interventions in humans with RA remains limited, and the underlying mechanisms are not yet fully understood. The present perspective reflects our currrent knowledge regarding fasting and CR, emphasising their impact on clinical outcomes, potential underlying mechanism and the sustainability of their effects. By exploring these strategies, we aim to provide a foundation for further research and highlight the need for well-designed clinical trials to determine the therapeutic efficacy and long-term feasibility of fasting and CR in RA management.
Opposed Reviewers:	

The impact of Fasting and Caloric restriction on Rheumatoid Arthritis in Humans: A narrative review

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Keywords: Fasting; Nutrition; Rheumatoid Arthritis; Autoimmune diseases; Chronic diseases; Intermittent fasting

Abstract

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease affecting approximately 1% of the global population. It is characterized by swollen and painful joints eventually evolving into bone erosion and cartilage degradation and systemic inflammation, significantly reducing patients' quality of life. While modern pharmacological treatments often lead to symptom improvement, they are accompanied by substantial side effects, which can further impair patient wellbeing.

Dietary interventions, particularly fasting and caloric restriction (CR), have gained increasing attention as adjunctive strategies for RA prevention and treatment. Their anti-inflammatory potential and ability to modulate the gut microbiome render them an attractive option to accompany or modify medical treatment. However, high-quality research on fasting and CR interventions in humans with RA remains limited, and the underlying mechanisms are not yet fully understood.

The present narrative review reflects our current knowledge regarding fasting and CR, emphasising their impact on clinical outcomes, potential underlying mechanism and the sustainability of their effects. By exploring these strategies, we aim to provide a foundation for further research and highlight the need for well-designed clinical trials to determine the therapeutic efficacy and long-term feasibility of fasting and CR in RA management.

Introduction

Rheumatoid arthritis

Non communicable diseases (NCDs) are the leading cause of mortality in the Western world, with their incidence continuously increasing [1]. Among these, rheumatoid arthritis (RA) stands out as a chronic, systemic autoimmune disease affecting approximately 1% of the global population and 31.7 million individuals are estimated to be living with RA by 2050[2]. The disease also has a high socoio-econmical impact as in addition to indirect and direct medical costs, 30% of patients with RA will become work-disabled in the first 2-3 year after their diagnosis[3]. As is common in autoimmune conditions, RA shows a pronounced sex disparity: women are three times more likely to develop RA than men, with an increased susceptibility during menopause and the post-partum period [4]. Patients with RA commonly experience severe and chronic pain, stiffness, and other inflammatory comorbidities, which significantly diminish their quality of life[5].

The etiopathogenesis of RA is multifactorial and not yet fully elucidated. Genetic, immunological, environmental, and lifestyle factors contribute to its initiation, progression, and severity. Central to RA pathogenesis is the production of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), which trigger the autoimmune recognition of citrullinated proteins in the joints [6]. This process is accompanied by an upregulation of proinflammatory chemokines and cytokines, triggering and perpetuating local inflammation, synovitis and cartilage damage [7].

Immune cell recruitment further exacerbates inflammation. CD4+ T cells, B cells, natural killer (NK) cells, dendritic cells (DCs) and mast cells infiltrate the synovium, releasing various proinflammatory cytokines. Of these, interleukin-6 (IL-6) and tumour necrosis factor- α (TNF-α) plays pivotal roles in disease progression and joint destruction [6,8].A hallmark of the disease is the pro-inflammatory loop, further promoting inflammation and immune system activation in the joints and thereby inducing bone erosion and cartilage degradation[9].

Several environmental and lifestyle factors contribute to RA development. Among the most prominent is smoking, which promotes citrullination at local mucosal sites, thereby increasing RA risk[10]. Another significant factor is diet, particularly via its influence on the gut microbiome[11]. Dysbiosis, an imbalance in the gut microbiome disrupting the health-promoting harmony of eubiosis, is increasingly recognized as a contributor to immune dysfunction in RA. An increase in Firmicutes and Proteobacteria, including

Aggregatibacter actinomycetemcomitans, *Prophyromonoas gingivalis and Akkermansia muciniphila as well as a decrease in Bacteroides,* have been associated with increased RA susceptibility through mechanisms like metabolite secretion, facilitation of citrullination, biomimicry and heightened gut permeability[11,12] (Figure 1).

Although advancements in pharmacological treatments have significantly improved the management of RA, a substantial proportion of patients remain non-responsive to therapy[13,14]. Moreover, the side effects of current medications, such as increased risk for infections, gastrointestinal side effects or cushingoid effects, can further impair quality of life[15]. This underscores the need for complementary therapeutic strategies aimed at symptom alleviation and disease prevention. Dietary interventions—particularly fasting and caloric restriction (CR)—have emerged as promising approaches due to their ability to confer anti-inflammatory effects and their potential to modulate systemic metabolism and the gut microbiome. Emerging research highlights the potential of these strategies to modulate inflammation, potentially via restoring microbiome balance.

Fasting and caloric restriction in RA

Fasting and CR have generated considerable attention for their potential benefits, in particular their anti-inflammatory effects.

Fasting refers to voluntary abstinence from caloric intake for specific periods, ranging from short-term (intermittent fasting) to prolonged fasting or fasting-mimicking diets, which are increasing in popularity in recent years due to the rising health and wellness culture in the industrialised countries. Notably, fasting differs from starvation, as fasting is intentional and followed by refeeding periods without additional dietary restrictions[16,17]. CR, in contrast, involves a sustained reduction in caloric intake to approximately 70% of a normo-caloric diet while maintaining sufficient macro- and micronutrient intake to prevent deficiencies. Unlike fasting, CR does not impose temporal restrictions on food consumption (Table 1).

Emerging evidence suggests that fasting and CR exert their beneficial effects through multiple mechanisms. Fasting induces essential metabolic and immunological pathways, that are critical for maintaining homeostasis and adapting to energy scarcity [18]. During regular energy consumption, ATP is primarily produced through glycolysis. However, during fasting, reduced glucose availability triggers the mobilization of energy from adipose tissue and protein stores [18,19]. This metabolic switch typically begins after 10-16

hours after the last caloric intake, depending on glycogen reserves and the composition of the previous meal [20].

Fatty acids (FAs) released from the adipose tissue are converted into fatty acyl CoA and subsequently increase the secretion of ß-hydroxybutyrate (BHB), a key ketone body [21]. BHB serves not only as an alternative energy source but also as a signalling molecule that regulates the expression of transcription factors such as sirtuins [20]. Sirtuins play vital roles in modulating anti-inflammatory responses, metabolic regulation and protection against oxidative stress, which may explain the several beneficial effects of fasting[22],[23].

The health benefits associated with fasting are wide-ranging and extend beyond metabolic improvements. They include enhanced psychological wellbeing[24,25], neuroprotective effects[26], improved metabolic health[27], symptom reduction in autoimmune diseases [26] as well as anti-cancer properties and support during chemotherapy in humans[24-26,28]. These effects are likely mediated through fasting's ability to reduce inflammation, restore metabolic balance and modulate immune responses, including those relevant to RA pathophysiology.

Results

Fasting interventions have emerged as a potential complementary therapy for RA, showing transient but significant benefits on disease activity and inflammatory markers. The studies included in this review (Table 2) consistently demonstrated clear physiological effects in relation to inflammation, metabolic processes, and microbiome dynamics, underscoring its relevance as a possible therapeutic approach. The interventions led to tangibleimprovements in clinical and inflammatory markers during fasting, reinforcing the reproducibility and reliability of its benefits for RA management. Although four of them utilized overlapping cohorts subjected to fasting interventions, each examined different aspects, such as RA disease activity markers, microbiome changes, and immunoglobulin glycosylation[29-33].

Sundqvist et al. [34] reported that a 10-day fasting period significantly reduced disease activity scores, including joint inflammation and erythrocyte sedimentation rate (ESR). These effects were linked to decrease in intestinal permeability, clearly indicative of a gut barrier-related mechanism. Similarly, Uden et al. [35] observed substantial clinical improvements in joint status and reductions in ESR during fasting, accompanied by enhanced neutrophil bactericidal capacity, which may contribute to modulating inflammation.

Fraser et al. [36] identified potential immunological mechanisms, involving the immune system, more specifically reporting a 37% reduction in serum interleukin-6 (IL-6) levels after a 7-day fasting intervention, which correlated with reduced C-reactive protein (CRP) levels and disease activity. Kjeldsen-Kragh et al. [33] further highlighted that fasting significantly reduced agalactosyl IgG levels, with these reductions correlating withclinical improvement.

Microbiome-related changes during fasting were investigated by Peltonen et al. [30] and Abendroth et al. [37], with findings indicating increased acetate levels and shifts in shirt-chain fatty acid (SCFA) profiles, particularly higher levels of acetate in fasting individuals. These microbiome changes were associated with significant clinical improvements, including reductions in the Disaese Activity Score (DAS-28), joint pain, and ESR, as well as improved visual analog scale (VAS) scores for pain perception. The gut-mediated benefits of fasting could involve several mechanisms: increased acetate may enhance intestinal epithelial barrier integrity, reducing systemic inflammation by lowering the translocation of bacterial endotoxins. Additionally, SCFAs like butyrate and propionate have known anti-inflammatory properties, such as inhibiting nuclear factor kappa B (NF-KB) pathways and reducing pro-inflammatory cytokines like IL-6 and TNF-α. These mechanisms collectively highlight the potential of fasting to modulate gut microbiota and contrivute to systemic anti-inflammatory effects (Figure 2).

In addition to its benefits, fasting isassociated with very detrimental few side effects. Gastrointestinal discomfort, including nausea, bloating, and diarrhea, is commonly reported, particularly when preparatory laxatives are used [35,37,38]. Fatigue and weakness are also observed, likely due to caloric deprivation and metabolic adaptations. These effects underscore the need for the close monitoring of patients during fasting interventions.

Kjeldsen-Kragh et al. [39]demonstrated significant reductions in inflammatory markers, including IgM RF, leukocyte count, and complement components C3 and C4, correlating these changes with improved clinical outcomes. Sköldstam et al. [38] confirmed fasting's efficacy in reducing pain, stiffness, and inflammation-related markers, such asα-1-acid glycoprotein, though these benefits were temporary.

Discussion

Our review synthesizes evidence highlighting fasting's potential to modulate RA symptoms through metabolic, immunological, and microbiome-related mechanisms. The studies consistently demonstrated

significant reductions in disease activity markers such as ESR, CRP, and IL-6, accompanied by improvements in clinical symptoms, including reductions in joint swelling, pain, and stiffness [34,35,40]. However, these results should be interpreted with caution due to the variability in study designs and fasting protocols.

One of the primary mechanisms of fasting appears to involve the generation of ketone bodies, such as BHB, which not only serve as alternative energy substrates but also function as signaling molecules. BHB potentially modulates inflammatory pathways by inhibiting the NLRP3 inflammasome and upregulating antioxidant responses via nuclear factor erythroid 2-related factor (Nrf2) amongst others. Additionally, BHB has been associated with enhanced mitochondrial function, reduced oxidative stress, and altered immune cell activity. These multifaceted yet convergent effects emphasize the critical role of BHB in fasting's anti-inflammatory benefits[36,41]. However, the extent to which BHB accounts for the observed clinical benefits remains uncertain, as the beneficial changes were not observed when following a ketogenic diet. A more complex immunomodulatory mechanism of fasting is suggested, involving systemic adaptations that require more comprehensive exploration.

As previously mentioned, the gut microbiome has gained recognition as a key player in RA pathogenesis. Several opportunistic pathogens identified in patients with RA have been associated with increased secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, as well as the activation of inflammatory cascades, including the NF-κB pathway. These signaling pathways can be activated by microbel-associated molecular patterns (MAMPs) and danger-associated molecular patterns (DAMPs), such as lipopolysaccharides and reactive oxygen species amongst others. The interplay between these microbial signals and host immune responses further exacerbates systemic inflammation in genetically predisposed individuals. Several studies have proposed fasting-induced alterations in gut microbiota composition, including changes in SCFA production[30,37]. Although the specific microbiota changes vary across studies, the consistent modulation of the gut microbiome highlights fasting's potential as a tool for restoring microbiome balance in RA patients. Dysbiosis-driven disruptions in intestinal homeostasis lead to increased permeability, increasing exposure to MAMPs and pathogen-associated molecular patterns (PAMPs). These molecules activate pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), triggering systemic inflammation and contribute towards autoimmune responses. Additionally, DAMPs released during fasting may transiently modulate the immune system and inflammatory processes[42,43]. While promising, the complexity of these interactions underscores the need for advanced studies to dissect causal relationships between microbiome alterations and RA pathogenesis.

In addition to effects directly modulated by the gut microbiome, the observed reductions in agalactosyl IgG and IL-6 during fasting provide insights into fasting's immunomodulatory effects[30,36]. However, these changes were not sustained post-refeeding, underscoring the transient nature of these benefits[33]. This temporary nature highlights a critical limitation of fasting interventions without a maintenance diet. Strategies to sustain these improvements, such as additional dietary and lifestyle adaptations, therefore warrant further exploration.

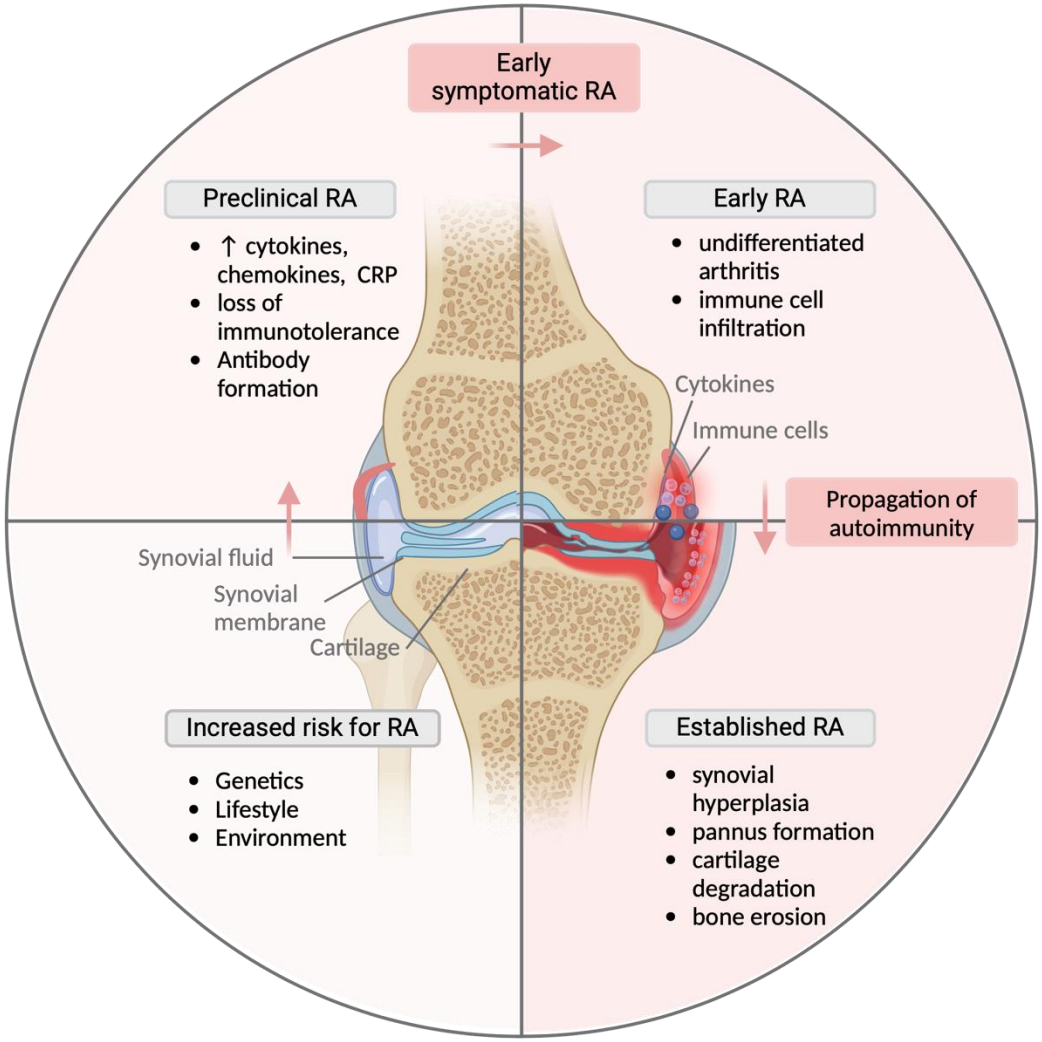
Despite its benefits, fasting is not without risks. Gastrointestinal discomfort, including nausea, bloating, and diarrhea, as well as fatigue, are frequently reported across studies, particularly during prolonged fasting periods or when preparatory laxativesused[44]. Nutritional deficiencies and rapid weight loss add complexity to fasting's application, especially for vulnerable populations such as those with comorbidities or advanced disease[45]. These issues underscore the importance of careful patient selection, ongoing medical supervision, and individualized intervention strategies to mitigate potential complications[46]. Additionally, fasting can impose psychological stress, manifesting as irritability or mental fatigue, and may create challenges in social contexts where shared meals are integral. Addressing these adherence barriers is essential for the successful integration of fasting into clinical care settings. An individualized approach to fasting interventions may offer enhanced safety and efficacy, particularly for patients with specific metabolic conditions, comorbidities, or medication regimens. Tailoring fasting parameters, such as the timing, duration, and frequency of caloric restriction, should further optimize its benefits while mitigating risks. Furthermore, emerging evidence suggests that personalized fasting protocols could be based on the composition of the baseline microbiome, allowing for targeted modulation of gut dysbiosis and immune responses[44-46].

Conclusion

Fasting represents a promising complementary therapy for RA, particularly for patients seeking alternatives to pharmacological treatments or experiencing treatment resistance. However, the literature on fasting in RA is scarce and presents mostly short-term studies, with limited exploration of long-term outcomes. Fasting protocols vary widely in terms of duration, dietary composition during refeeding, and overall study design. This lack of standardization complicates comparisons across studies and limits the generalizability of findings. Additionally, many earlier studies relied on less sophisticated analytical methods, leaving significant gaps in our understanding of fasting's molecular and cellular mechanisms.

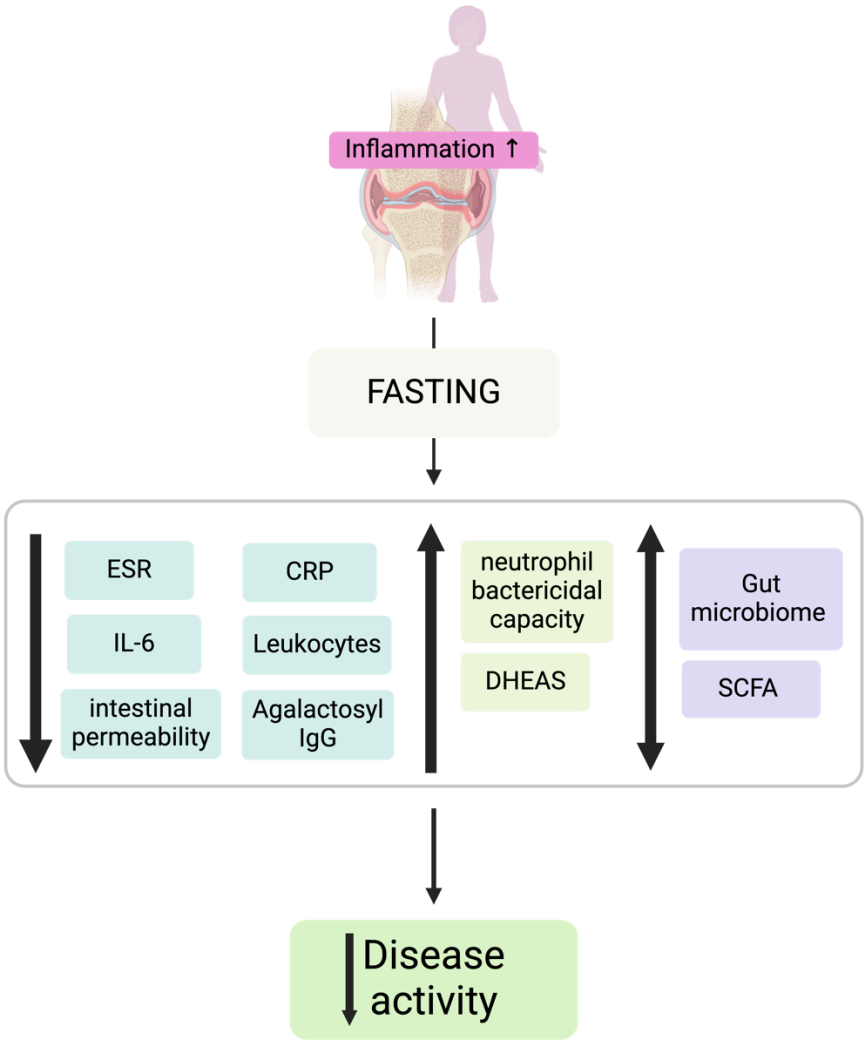
Thus, fasting's ability to rapidly reduce systemic inflammation offers a valuable addition to RA management. However, integrating fasting into clinical practice requires addressing its temporary effects and ensuring patient safety, considering the above-mentioned possible side effects. To maximize therapeutic potential, it is essential to develop sustainable and individualized fasting regimens that maintain the longer-term beneficial effects. Future research should focus on standardizing fasting protocols and exploring the interplay between fasting, the microbiome, and immunometabolism using advanced techniques such as metagenomics, transcriptomics, and metabolomics to elucidate molecular pathways.

More research is needed to optimize fasting protocols, investigate long-term effects and explore personalized approaches to maximize its therapeutic potential in RA. The currently ongoing ExpoBiome study is studying the effect of prolonged fasting followed by a maintenance diet, consisting of 12 months of time-restricted eating in patients with RA and patients with Parkinson's disease and will elucidate complex underlying mechanisms of this intervention and its beneficial health outcomes.



223

224 Figure1: Different stages of rheumatoid arthritis. This figure illustrates the different stages of RA, moving
225 from the first phase with a higher susceptibility to develop RA, to a first involvement of the immune
226 system at the preclinical stage, and the appearance of undifferentiated arthritis during the phase of early
227 RA, followed by propagation of autoimmunity leading to established RA. Created in BioRender. Hansen, B.
228 (2024)



229

230 Figure 2: Summary of beneficial fasting effects observed in patients with rheumatoid arthritis. Created in

231 BioRender. Hansen, B. (2024)

Table 1: Different types of fasting and caloric restriction.

Type of fasting	Duration / re-occurrence	Energy intake
Prolonged fasting / Long-term fasting	> 4 days – several weeks	200 – 350 kcal / day
Short-term fasting	2-4 days	200 – 350 kcal / day
Intermittent Fasting	Alternation of fasting periods (≤ 48 h) and ad libitum food intake	0 kcal alternating with ad libitum
Alternate day fasting	Total fasting or modified fasting on alternate days	0 kcal alternating with ad libitum
Time restricted eating	Periodic total fasting ≥ 14 h/day	No food intake during fasting, ad libitum during eating phase
Periodic fasting	Any type of fasting repeated at regular intervals	Depends on fasting method applied

Caloric restriction (CR)	undefined	~ 70% of normocaloric intake (avoiding malnutrition)
Fasting mimicking diet (FMD)	5 days of FMD with 1 to 6 cycles per year	800 – 1100 kcal

Table 2: Summary of Studies on RA and Fasting regime.

Paper	*Intervention*	*Duration*	*Participants (n)*	*Age (mean)*	*Gender*	*Outcome*
Sköldstam et al., 1979[38]	Fasting and lactovegetarian diet	7-10 days fasting + 9 weeks lactovegetarian diet	26 (Diet: 16 Control: 10)	Diet group: 52 (35-66). Control group: 54 (43-65)	19 females (10 diet group, and 9 control group) 7 males (6 diet group and 1 control group)	Fasting led to temporary improvements, no direct microbiome analysis. The lactovegetarian diet had limited effect.

Sundqvist et al., 1982[34]	Fasting and lactovegetarian diet	10 days fasting + 1-week lactovegetarian diet	10 (Diet: 5, Control: 5)	Not specified	Not specified	Fasting decreased intestinal permeability and disease activity. No direct microbiome analysis, but suggested a microbiome influence due to improved gut barrier function
Uden et al., 1983[35]	Fasting + normal food intake (cross-over study)	7 days + 7days	13	42 (24-60)	Females	Fasting reduced joint inflammation, ESR, and improved neutrophil bactericidal capacity. No direct microbiome findings
Kjeldsen-Kragh et al., 1991[29]	Fasting and vegetarian diet	7-10 days fasting + 3.5 months gluten-free vegetarian diet + 9 months lactovegetarian diet	53 (Diet: 27, Control: 26)	Diet group: 56 (38-78). Control group: 53 (26-63)	45 females (24 diet group, and 21 control group) 8 males (3 diet group and 5 control group)	Fasting improved disease activity markers (ESR, CRP); vegetarian diet sustained benefits. Controls showed no significant improvements. No direct microbiome analysis.
Peltonen et al., 1994[30]	Fasting and vegetarian diet	7-10 days fasting + 3.5 months gluten-free vegetarian diet + 9 months	53 (Diet: 27, Control: 26)	Diet group: 56 (38-78). Control	45 females (24 diet group, and 21 control group)	Significant changes in intestinal flora correlated with RA symptom improvement

		lactovegetarian diet		group: 53 (26-63)	8 males (3 diet group and 5 control group)	
Kjeldsen-Kragh et al., 1995[31]	Fasting and vegetarian diet	7-10 days fasting + 3.5 months gluten-free vegetarian diet + 9 months lactovegetarian diet	53 (Diet: 27, Control: 26)	Diet group: 56 (38-78). Control group: 53 (26-63)	45 females (24 diet group, and 21 control group) 8 males (3 diet group and 5 control group)	Reduction in P. mirabilis antibodies and improvement in disease activity in diet responders
Kjeldsen-Kragh et al., 1995[39] *Based on the abstract. Full article*	Fasting and vegetarian diet	7-10 days fasting + 3.5 months gluten-free vegetarian diet + 9 months lactovegetarian diet	53 (Diet: 27, Control: 26)	Diet group: 56 (38-78). Control group: 53 (26-63)	45 females (24 diet group, and 21 control group)	Elevated antibody activity against dietary antigens was observed in RA patients, but it did not correlate with clinical outcomes, suggesting food-related immune responses are unlikely to be involved in RA pathogenesis.

not available.						
Kjeldsen-Kragh et al., 1995[32]	Fasting and vegetarian diet	7-10 days fasting + 3.5 months gluten-free vegetarian diet + 9 months lactovegetarian diet	53 (Diet: 27, Control: 26)	Diet group: 56 (38-78). Control group: 53 (26-63)	45 females (24 diet group, and 21 control group)	Decrease in inflammatory markers, leukocyte counts, and complement activity linked to diet. No direct microbiome analysis.
Kjeldsen-Kragh et al., 1996[33]	Fasting and vegetarian diet	7-10 days fasting + 3.5 months gluten-free vegetarian diet + 9 months lactovegetarian diet	53 (Diet: 27, Control: 26)	Diet group: 56 (38-78). Control group: 53 (26-63)	45 females (24 diet group, and 21 control group) 8 males (3 diet group and 5 control group)	Decrease in agalactosyl IgG correlated with clinical improvement post-fasting, but not after vegetarian diet period

Fraser et al., 2000[40]	Fasting or ketogenic diet	7-day fasting vs. ketogenic diet	23 (Fasting: 10, Ketogenic diet: 13)	Fasting: 49 (31-65); Ketogenic diet: 44 (25-69)	Fasting: 9 females, 1 male. Ketogenic diet: 12 females, 1 male	Fasting reduced IL-6 and improved disease activity; both interventions increased DHEAS
Michalsen et al., 2005[47]	Mediterranean diet vs 8-day intermittent fasting	2 weeks + 3-month follow-up	51 (RA: 16, FM: 35)	49.4 ± 14.3 (MD), 57.7 ± 6.5 (Fasting)	MD: 7 females, 0 male. Fasting: 9 females, 0 male	No significant changes in fecal flora or sIgA; clinical improvement in RA observed with fasting (p=0.09)
Abendroth et al., 2010[37]	Mediterranean diet and fasting	7 days fasting or MD	50 (Fasting: 22, MD: 28)	Fasting: 55.7, MD: 60	Fasting: 21 females, 1 male. MD: 26 females, 2 males	Significant reduction in DAS-28 for both groups, more pain reduction in fasting group. Microbiota alterations were observed with both interventions. Alterations in SCFA in fasting group causing increase of acetate levels.
Hartmann et al., 2023[48]	Fasting + plant-based diet (PBD) vs anti-inflammatory diet (AID)	7 days (fast) + 11 weeks (PBD) vs 12 weeks (AID)	41 PBD: 24 AID: 17	Not specified	Females	Both diets had comparable impacts on nutrient intake and RA symptoms

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Author contributions

The authors' responsibilities were as follows—BH, MSC: conceptualized the research approach, planned and drafted the manuscript outline; BH, MSC: wrote the paper; LS, AK: contributed to literature research; JGS, PW: reviewed and edited the manuscript; and all authors: read and approved the final manuscript.

Conflicts of Interest and Funding Disclosure

None.

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4. Discussion

In contrast to the perspective on the effects of fasting in NDs, we were able to compile several clinical studies examining fasting in patients with RA. However, despite the longstanding interest in this approach, it is striking how little progress has been made in fully understanding its mechanisms and long-term benefits, even four decades after the first results were published. Given the immense potential regarding dietary interventions, this highlights the pressing need for a more in-depth analysis of patient samples, as well as the rigorous design and implementation of additional nutritional clinical trials. Such studies will be crucial for elucidating the precise biological pathways influenced by fasting and for optimizing dietary strategies tailored to the needs of RA patients.

C. Manuscript III:

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)

Hansen B, Laczny CC, Aho VTE, Frachet-Bour A, Habier J, Ostaszewski M, Michalsen A, Hanslian E, Koppold DA, Hartmann AM, Steckhan N, Mollenhauer B, Schade S, Roomp K, Schneider JG, Wilmes P. 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). BMJ Open. 2023 Aug 18;13(8):e071380. doi: 10.1136/bmjopen-2022-071380. PMID: 37597865; PMCID: PMC10441058.

1. Contribution

As the first author of this manuscript, my contributions to this research paper include the study design and protocol preparation, the writing of the manuscript and coordination of the editing and publishing processes as well as the figure creation.

2. Background and introduction

The systemic chronic auto-immune disease RA has been introduced in detail in IV. This NCD affects almost 1% of the global population and has severe consequences on the daily lives of the patients and decreases the quality of life significantly. Another NCD that has been continuously increasing during the past decades is PD. This condition is a chronic, progressive neurodegenerative disease marked by the loss of dopaminergic neurons in the substantia nigra and is associated with the aggregation of a-synuclein, leading to the formation of Lewy-bodies, accumulating in the neurons. The dopamine depletion eventually leads to a disruption in neurological pathways, leading to the characteristic motor symptoms of PD, including bradykinesia, tremor and rigidity. Additionally, PD does not only affect motor skills but does also lead to non-motor symptoms such as depression, sleep disturbance and cognitive impairment. Similarly to RA, there is currently no cure available for PD. Several different treatment options offer a reduced disease progression and symptom alleviation, however, the extent to which the disease can be slowed down varies and several patients do not respond to medication at all. In addition, the commonly prescribed treatment options come with multiple side effects. Fasting, as already elaborated in greater detail in the introduction, has been reported to have several beneficial health effects, including an overall reduction in inflammation and an amelioration of the gut microbiome composition. As both patients with RA and PD have been reported to show differences in their intestinal microbiome compared to healthy controls, a shift in the latter might be a promising alternative intervention strategy.

Prolonged fasting followed by a vegetarian diet has been reported to be beneficial in patients with RA, however the underlying mechanisms could not be deciphered yet[128]. For patients with PD, no such study has been previously done and the ExpoBiome study is the first to look in depth into the differences and similarities between auto-immune and neurodegenerative disease compared to healthy controls as well as at the different impact PF and IF have on RA and PD.

The ExpoBiome study encompasses several different aspects, including a cross-sectional study, a longitudinal study, three different cohorts consisting of patients with RA, patients with PD, and healthy controls and multiple different experiments and analyses. A broad sample collection allows the detailed analysis of different aspects of both diseases and compare these findings to baseline data from HC. The aim of this paper was to streamline all the different aims and planned experiments with the according planned analyses for future reference.

3. Manuscript

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)

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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 (PF) 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, specialised clinical sites. For the longitudinal part, 30 patients with PD and 30 patients with RA undergo 5–7 days of 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 NCT04847011.

STRENGTHS AND LIMITATIONS OF THIS 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.
⇒ This study will identify novel microbiome-derived common and disease-associated molecules involved in immune system modulation in two major chronic diseases: rheumatoid arthritis (RA) and Parkinson's disease (PD).
⇒ This study aims at also identifying novel targeted pathways to control chronic inflammatory conditions in the future.
⇒ A limitation is the heterogeneity of the cohorts regarding age and sex, which is due to the prevalence of the diseases: RA is more common in women, while PD is more common in men and has a later disease onset.
⇒ A bias exists in choosing RA and PD as chronic disorders to study immunophenotypes although generalisable results are targeted.

INTRODUCTION

The human microbiome is emerging as a key driver of various diseases through its complex of distinct yet connected biomolecules (referred to as the '*expobiome*').[1 2] The expobiome comprises a diverse set of nucleic acids, polypeptides and metabolites which, in the gut alone, are present in substantial concentrations.[1] However, the specific

interactions between gut-borne microorganisms and host (patho)physiology remain largely unknown. Although host genetics shape the composition of the gut microbiome, the latter is particularly influenced by non-genetic factors such as lifestyle and diet.[3 4] Therefore, the microbiome is a plausible target to modify health outcomes.

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

Rheumatoid arthritis (RA) and Parkinson's disease (PD) are two specific examples representing dysregulated microbiome-immune system interactions.[7 8] RA is a multifactorial, chronic and systemic autoimmune disease, primarily affecting the lining of the synovial joints with a higher risk and younger age for disease onset in women and a global prevalence of 1%.[9 10] The exact disease pathogenesis is still unclear and no cure for RA currently exists. In addition to the common local articular symptoms of RA, systemic comorbidities can affect the vasculature, metabolism and bones.[11] Besides various environmental risk factors, for example, smoking and a Western diet, the host microbiome is associated with the pathophysiology of the disease.[12] The diversity of the gut microbiome has been reported to be decreased in individuals with RA, compared with the general population, and is correlated with disease duration, activity and autoantibody levels.[13 14] Studies in murine models also report that autoimmune arthritis is strongly attenuated under germ-free conditions.[15] The introduction of specific bacteria, for example, segmented filamentous bacteria, into germ-free animals or oral infection with *Porphyromonas gingivalis* drive autoimmune arthritis through activation of T helper cells.[15] Several different taxa, including *Prevotella copri*, *Lactobacillus* spp and *Colinsella* spp are enriched in the gut microbiome of patients with RA and correlate positively with disease markers, for example, immunoglobulins IgA and IgG, while other taxa like *Haemophilus* spp and *Faecalibacterium* spp are typically found at lower abundances in patients with RA compared with healthy individuals.[13 16 17] Alterations of the gut microbiome may, therefore have an important impact on RA pathophysiology.[12]

PD affects 0.4%–2% of the population over 65 years worldwide and is the second most common progressive neurodegenerative disease with men being 1.5 times more likely to be affected than women.[18] Cardinal symptoms are not only motor deficiencies such as tremor and rigidity but also include a wide range of non-motor symptoms, such as hyposmia, depression, insomnia or cognitive impairment, severely impacting patients' quality of life.[19] Aggregations of the protein a-synuclein in the dopaminergic substantia nigra represent the main neuropathological manifestations.[20] PD-associated loss of dopaminergic neurons involves mechanisms of inflammatory and autoimmune responses with microglial activity as a major driver.[21] Dysbiosis of the gut microbiome has been associated with the characteristic motor deficits and pathophysiological changes in the enteric and central nervous systems in animal studies. Increased relative abundances of the genera *Akkermansia, Bifidobacterium, Lactobacillus* and *Methanobrevibacter* and decreased abundances in *Faecalibacterium* and *Roseburia* have been reported.[22 23] Two recently published clinical trials with prebiotic supplementation in PD observed a shift in gut microbiome composition, an increase in short-chain fatty acids (SCFA) and a reduction in non-motor symptoms.[24 25] Most patients with PD suffer from gastrointestinal symptoms such as constipation and irritable bowel syndrome-like symptoms.[26] The gut-brain axis, for example, by-products produced by the gut microbiome, may contribute to the production of a-synuclein aggregates in the enteric nervous system.[27] In addition, increased intestinal permeability[28] as driver for enteric inflammation occurs in PD and substantiates a role of peripheral inflammation in the initiation and the progression of the disease.[29]

One factor with known major impact on the gut microbiome and on chronic diseases is diet.[7] Dietary approaches as fasting have already been used by Hippocrates in the fifth century before Christ and have been applied ever since by numerous medical schools to treat acute and chronic diseases.[30–32] Various practices of caloric restriction through fasting have repeatedly shown remarkable health benefits.[33 34] Maifeld *et al* found that a 5-day fast followed by a modified Dietary Approach to Stop Hypertension (DASH), with additional emphasis on plant-based and Mediterranean diets, reduced systolic blood pressure, body mass index (BMI) and the need for antihypertensive medications at 3 months post intervention compared with DASH alone.[35]

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

These improvements can, however, typically only be maintained for a limited period of time, and the symptoms can reappear after reintroduction of the patients'

standard diet. Hence, protocols to sustain these beneficial effects are of utmost importance. In mouse models of PD, intermittent fasting (IF) has led to several improvements including decreased excitotoxicity, reduced neurodegeneration and protection against autonomic dysfunction, motor and cognitive decline.[38]

IF and prolonged fasting (PF) may have potent immunomodulatory effects, which may partially be mediated by the gut microbiome and the fasting-induced alterations of the latter.[39] These microbial shifts include upregulation of not only *Akkermansia muciniphila*, *Bacteroides fragilis*, other *Bacteroides* spp, Proteobacteria and butyric acid producing *Lachnospiraceae* but also *Odoribacter*, which is negatively associated with blood pressure.[35 40] Interestingly, an overall decrease in the Firmicutes/Bacteroidetes ratio could be observed, a high ratio is commonly associated with several pathologies, including RA.[41]

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

The impact and importance of the gut microbiome on human physiology and its potential modifications by nutrition and dietary patterns have been underestimated for centuries.[43] Reasons may include missing standardised therapeutic protocols, the interindividual variability not only in the response to fasting, lack of knowledge about possible adverse effects and difficulties in the interpretation of underlying mechanisms seen in clinical trials but also in the comparably low potential for achieving economic revenue or scientific impact.[8]

Modern experimental approaches and computational integration allow a multilayer analysis of digestive processes in low caloric settings, including the gut microbiome.[44] These technological developments also permit a closer investigation of the link between the immune system and severe caloric restriction.

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

METHODS AND ANALYSIS

Study objectives

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

Study design

The ExpoBiome cohort consists of 180 adult individuals, meeting the exclusion and inclusion criteria (table 1), for the cross-sectional study (objectives 1 and 3) and 60 adult individuals for the longitudinal study (objectives 2 and 3). There are five different arms in total: (1) RA—cross-sectional arm (60 patients), (2) PD—cross-sectional arm (60 patients) and (3) healthy controls—cross-sectional arm (60 patients), (4) RA—longitudinal arm (30 patients), (5) PD—longitudinal arm (30 patients) (figure 1).

At the first visit (T0), patients answer several questionnaires, and blood, urine, saliva and stool samples are obtained (box 1).

The longitudinal arms (4) and (5) undergo a 5–7 day PF with a dietary energy supply of maximum 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 time-restricted eating (TRE) for a period of 12 months following the 16:8 pattern.[47] This means that food intake is allowed ad libitum for 8 hours, followed by 16 hours of fasting, where no food should be consumed. The intake of non-caloric beverages, for example, water, unsweetened tea or coffee is, however, allowed. The participants attend one follow-up visit (T2) during the PF and nine 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, for example, 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

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
► 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	► 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 (eg, kidney deficiency with creatinine >2 mg/dL) ► Existing vegan diet or fasting during the last 6 months ► Presence or suspicion of atypical PD (eg, early dementia, early autonomous dysfunction) ► Diagnosis of chronic inflammatory bowel diseases, coeliac 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 (eg, haemoglobin <10) ► BMI<18.5 ► Psychiatric illness that limits understanding of the examination protocol (unable to consent)

BMI, body mass index; PD, Parkinson's disease; RA, rheumatoid arthritis.

in Kassel, or by non-personal advertising strategies (eg, 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.

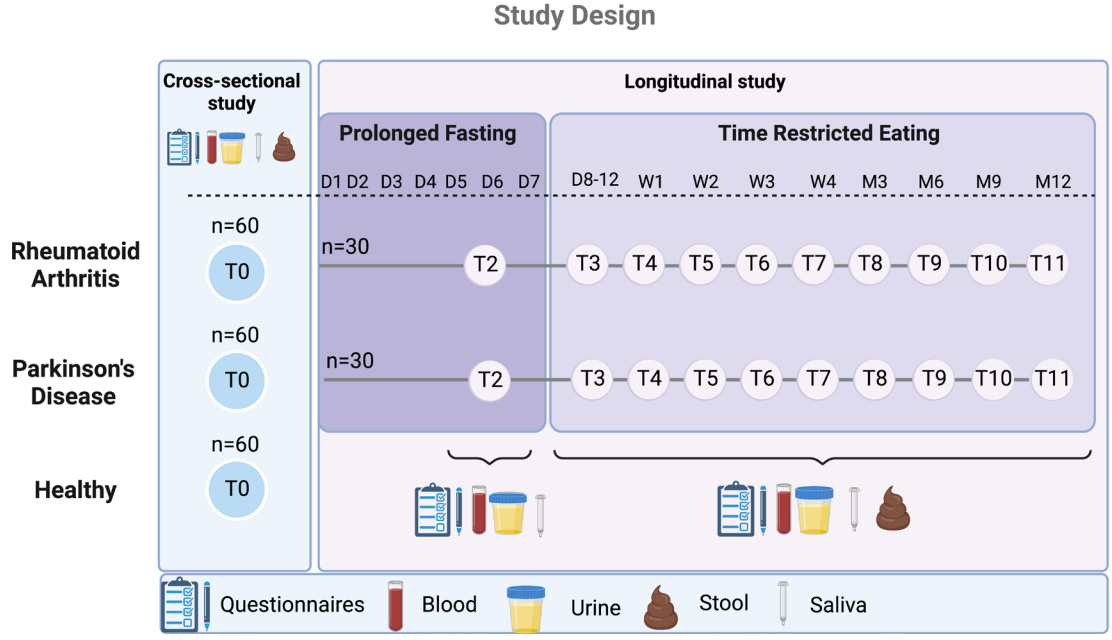

Figure 1 Study design. This figure illustrates the study design with five different arms in total, two of which continue with the longitudinal part of the study. Visits take place at the clinical sites at each timepoint and include the collection of the displayed samples. This image was generated using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

Box 1 Sampling procedures

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).

Questionnaires
Disease-specific
Parkinson's disease (PD)
⇒ Disease Activity Score.[72]
⇒ Parkinson's Disease Sleep Scale-2.[73]
⇒ Parkinson's Disease Questionnaire-39.[74]
⇒ Simplified Disease Index Score.[75]
⇒ Funktionsfragebogen Hannover.[76]
⇒ Movement Disorder Society Unified PD Rating Scale.[77]
⇒ Non-Motor Symptoms Questionnaire.[78]
⇒ Non-Motor Symptoms Scale.[79]
Rheumatoid arthritis
⇒ Disease Activity Score.[75]
⇒ Non-Motor Symptoms Questionnaire.[78]
⇒ Funktionsfragebogen Hannover.[76]
Dietary behaviour and lifestyle
⇒ Fasting experience, expectation, and intervention.
⇒ Lifestyle.
⇒ 24H-Food-recall.
⇒ Food Frequency Questionnaire.
General health and well-being
⇒ Health Assessment Questionnaire.[80]
⇒ Bristol Stool Scale.[81]
⇒ Quality of Life questionnaire.[82]
⇒ Hospital Anxiety and Depression Scale.[83]
⇒ Profile of Mood States.[84]

For patients with RA, the diagnosis has been made prior to the study by an experienced rheumatologist according to the European League Against Rheumatism 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 optimise 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 optimised 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 are 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.

Fasting dietary counselling

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

Medication

The medical treatments of the patients are monitored and documented with every clinical visit. The fasting intervention might necessitate temporary adjustments of several medications, for example, antidiabetic and antihypertensive drugs as insulin levels and hypertension will be reduced due to lack of food intake.[31]

Data collection

Sample and data collection are performed at the two clinical sites, Charité—Universitätsmedizin Berlin and Paracelsus-Elena Clinic (box 1).

Anthropometric data and questionnaires

The electronic data capture system REDCap,[52] a secure web-based application, is used to record all individual specific data. All data are stored on a secure server infrastructure at the host institution in Luxembourg. Weight, height, BMI, heart rate and blood pressure in sitting and standing position as well as waist–hip–ratio are determined at every visit. Dietary behaviour, sociodemographic measurements (age, sex, education level, employment status, marital status), family history, current and previous illness and comorbidities and current medications as well as disease-specific data, questionnaires about the well-being of the patients and data on the behavioural factors are collected at baseline, T6 (week 3), T9 (month 6) and T11 (month 12) (box 1). Questionnaires (24 hour-Food

Table 2 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.	Alanine Transaminase (ALT), 37°C
Eosinophils, %	Alkaline phosphatase, 37°C
Eosinophils, abs.	Aspartate Transferase (AST), 37°C
Erythrocytes	Bilirubin, total
Haematocrit	Cholinesterase
Haemoglobin	Cholesterol
HbA1c	Creatinine
Leucocytes	high-sensitivity C-reactive protein (hs-CRP)
Lymphocytes, %	Glucose, serum
Lymphocytes, abs.	Gamma-GT, 37°C
Mean corpuscular hemoglobin (MCH)	High density lipoprotein (HDL)-cholesterol
Mean corpuscular hemoglobin concentration (MCHC)	Low density lipoprotein (LDL)-cholesterol
Mean corpuscular volume (MCV)	Potassium
Monocytes, %	Sodium
Monocytes, abs.	Total protein
Neutrophils, %	Triglycerides
Neutrophils, abs.	Uric acid
Platelets	Urea/Blood Urea Nitrogen
Red cell distribution width (RDW)	Proteins—serum
Reticulocytes	Rheumatoid factor H 35.9
Reticulocytes	Hormones—serum
Reticulocytes, abs.	Insulin
	Thyroid stimulating hormone (basal)

Recall, Bristol Stool Scale) are answered at all visits by the study participants. Data storage, analysis and exchange are done only in pseudonymised fashion. The nutritional data are analysed using the Nutrilog V.3.20 software (Nutrilog SAS, Marans).

Blood samples and parameters

Blood samples are collected at each visit and immediately used for peripheral blood mononuclear cell (PBMC) isolation (T0), analysis by the study laboratory and centrifugation to freeze plasma samples at −80°C (T0–T11). A clinical standard laboratory report is generated after every visit for each study participant (table 2). In addition to routine blood parameters, anticitrullinated protein antibody, zonulin, fatty acid binding protein 2 and calprotectin levels are measured. Aliquots are securely stored to account for novel observations and testing of hypotheses.

Stool, urine and saliva samples

The samples listed in box 1 are collected at each visit, except for stool samples on T2 (fasting week) and immediately frozen and stored at −80°C. Stool characteristics are recorded at the time of the sampling. Faecal samples represent the main sample type for resolving the dynamic processes driven by microbiome in the gut. Also, as the gut microbiome is prone to diurnal fluctuations, the stool samples are collected in the morning, as far as possible.

Methods applied to samples

Biomolecular extractions

The collected stool samples undergo a biomolecular extraction procedure to allow isolation of concomitant DNA, RNA, proteins, peptides and metabolites from single, unique faecal water samples; this process involves cryomilling the samples in liquid nitrogen, disassociating metabolites from membrane and cell wall components in a solvent mixture of methanol, chloroform and water and lastly proteins and RNA extraction by a methanol/chloroform and phenol buffer.[53 54] Faecal water is recovered following centrifugation and filtration, at low speed or low flow, respectively, to avoid cell lysis. Nucleic acids are preserved by the addition of ribonuclease inhibitors and isolated by silica column-based techniques. This protocol involves the use of a robotic platform, ensuring a higher level of standardisation and reproducibility.[2]

Coupled MG and MT analyses

Prior to sequencing library preparation, internal standards are introduced to obtain quantitative sequencing data.[55] Contamination-free MG and MT data are generated, processed and analysed using the integrated meta-omics pipeline (IMP),[45] which incorporates preprocessing, assembly, gene annotation, mapping of reads, single nucleotide polymorphism calling, data normalisation as well as analyses of community structure and function in a fully reproducible software framework based on Docker. The MG and MT data are specifically screened for enrichments in genes and pathways with known immunogenic properties.[56] The extracellular biomolecules are linked to specific microbial populations based on the intracellular MG data.[57] In addition, the sequencing data are mapped against genomes of food components.[44] The quantitative data are also related to microbial population sizes to determine the contribution of the resolved microbial populations in stool to the extracellular DNA and RNA complements.[58]

Metaproteomics

For the metaproteomic analyses, filtration is used to separate extracellular peptides from the obtained (poly) peptides. The resulting smaller fractions are then desalted and analysed without proteolytic digestion via liquid chromatography (LC) and mass spectrometry (MS) on

an EasyNano-LC coupled online to a QExactive-Plus mass spectrometer (ThermoScientific, Waltham). The identification of ribosomal peptides is done with an integrated catalogue of MG and MT data, while the non-ribosomal peptides are identified using different tools, that is, MyriMatch, DirecTag as well as CycloBranch.[45 59 60] The metaproteomic data also allow identification of extracellular (poly)peptides with possible pathogenic functions including protein misfolding and molecular mimicry.[61 62]

Metabolomics

Metabolomic data are analysed using a combination of targeted and untargeted approaches.[44 54 63] This highlights the major metabolite classes produced by the gut microbiome with an effect on human physiology, including organic acids, SCFA, lipids, branched-chain fatty acids, branched-chain amino acids, vitamins, bile acids and neurotransmitters. Besides external compound calibration series for quantification and quality control samples to ensure data normalisation and data acquisition quality assessment, the metabolite extraction fluid is fortified with multiple internal standards to improve method precision and accuracy.[64 65] The data are compared with in-house databases and public mass spectral libraries to identify known metabolites. The metabolomic data complement the MG and MT data and, thus, allow further establishments of conclusive links to metabolic properties in the gut.

Deep immune profiling

Deep immune profiling is done using a recently established and optimised panel of metal-labelled antibodies together with cytometry coupled to MS, the Maxpar Direct Immune Profiling System (MDIPA). This approach allows the simultaneous quantification of 38 parameters on single cells. Whole blood is stained with the MDIPA kit and stabilised with Proteomic stabiliser Prot-1 (501351694, Smart Tube, Las Vegas) before storage at −80°C. The quantified immune cells included in the MDIPA panel are CD3+, CD4+, CD8+, monocytes, dendritic cells, granulocytes, mucosal-associated invariant T cells (MAIT), T cells, natural killer (NK) and B cells.[66] Cytokine expression profiles are analysed on blood plasma using the Human Luminex performance Cytokine Panel (R&D Systems Europe, Abingdon), measuring CCL3, CCL4, CCL5, GM-CSF, IL-1b, 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-b, Galectin-1, IFN-g and TNF-a.[56]

Gut-on-a-chip models

PBMCs isolated from T0 blood samples are co-cultured with gut-derived microbes under physiologically representative conditions using the gut-on-a-chip model HuMiX.[67] This model of the human gastrointestinal interface allows the investigation of the interactions between immune, epithelial and bacterial cells and specifically the response to fasting in personalised in vitro models.

The expobiome MAP

The Expobiome Map (https://expobiome.lcsb.uni.lu) illustrates the diverse complex of microbial immunogenic molecules, including nucleic acids, (poly)peptides, structural molecules, and metabolites. The interactions between this “expobiome” and human immune pathways are encoded in the context of chronic diseases.[1] The ExpoBiome Map is visualised using the MINERVA Platform.[68] Clicking on different elements on the map reveals factors they affect and are affected by, allowing an easier navigation through the complex relationships between individual microbiome components in relation to human disease. The multi-omics data generated in the present study will be integrated with the Map.

Exploratory analysis of novel host-microbiome interactions

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

Outcome parameters

Primary outcome

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

Secondary outcome measures

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

Sample size and power calculation

A power calculation using pilot MT data based on faecal extracellular RNA samples was performed to determine the number of subjects to be recruited for the ExpoBiome project. The obtained relative abundances of genera were used for the calculation of the required sample size per group. The power calculation was based on the algorithm as described by Tusher, Tibshirani, and Chu.[69] To achieve a power of 90% (at α=0.05), a total of 50 individuals per group (RA, PD, healthy controls) must be analysed. Considering any possible dropouts, 20% additional subjects are recruited, resulting in a total number of 180 individuals, that is, 60 per group. For the longitudinal part, a subset of 60 adult individuals (30 patients with PD and 30 patients with RA) are selected, based on their ability and willingness to participate in the longitudinal part of the study (12 months follow-up). The selected number of participants for the longitudinal study is based on feasibility due to the complexity and high costs of the

clinical trial. The total number of subjects in the longitudinal study can be smaller, as each individual serves as their own control.

Adverse events

There are no major risks expected for participants. Minor common adverse effects of PF might include headaches, nausea, insomnia, back pain, dyspepsia and fatigue.[70] Any occurring adverse events are recorded at each visit in REDCap.[52] Serious adverse events are communicated to the study coordinator and principal investigator within 24 hours of their report.

Data management, monitoring, analysis and evaluation of data

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

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

Different statistical tests are performed according to the nature of the data. A premature termination of the study is not envisaged; therefore, no interim analysis is done. Different correlation measures are applied, including Spearman correlation, mutual information on discretised data, distance correlation, maximum information criterion, local similarity analysis and the bioenv approach. Comparison across all omic levels allows identification of common and disease-specific signatures. Multivariate machine learning is used to link different data features to observed patterns. For additional confounding factors, especially in the cross-sectional study, multivariate statistical analysis will be performed. These factors will be accounted by including confounders in the analysis, for example, as covariate in the statistical models.

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

The Standard Protocol Items: Recommendations for Interventional Trials checklist was used to write this protocol.[71]

Trial status

The recruitment for the ExpoBiome study started in April 2021 and is currently ongoing. All study participants should be recruited by the end of 2022. The sample collection will take place from 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 21–001A ExpoBiome). The results of this study will be disseminated through peer-reviewed publications, scientific presentations as well as press releases and social media postings (Twitter, LinkedIn). Study participants will be contacted and informed by the respective clinical sites about the outcome and results of the study, once the data analysis has been completed (dissemination phase).

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4. Discussion

The ExpoBiome study represents a groundbreaking initiative to revolutionize our understanding in several domains where evidence is currently lacking. By comprehensively analyzing data across diverse populations, ExpoBiome aims to uncover critical insights into the role of these factors in non-communicable diseases (NCDs), including areas that have been historically under-researched.

Therefore, the publication of a clearly structured study protocol is crucial. This protocol serves as a guideline that details the study's design, methodology, and analysis plan. This level of documentation and transparency ensures that the research process is replicable and provides the groundwork for achieving reliable and reproducible results. By adhering to a predefined structure, bias can be minimized, and systematic approaches can be followed for each phase of the study. This allows for more precise data collection and analysis, which is vital for drawing valid conclusions.

In addition to providing clear instructions for possible reproductions of the study, the publication of study protocols also guarantees scientific transparency and accountability. The early visibility of the study protocol, before publication of any results, helps prevent selective reporting and allows for constructive criticism, enabling the scientific community to comment on potential methodological flaws, improving the study's credibility.

Furthermore, study protocols can also serve as a resource for developing new studies, allowing scientists to learn from and improve on prior methodologies. They also encourage standardization across studies, particularly in clinical trials, where consistent methodologies are essential for comparing results across different populations and settings. This standardisation is crucial and mostly lacking in clinical intervention trials, especially in nutrition research.

Structured study protocols and their publication play a foundational role in advancing reliable, transparent, and impactful scientific research. This study stands to set a new standard in research methodology, providing the comprehensive evidence needed to address the pressing health challenges of our time.

D. Manuscript IV:

Immunophenotyping of patients with rheumatoid arthritis reveals difference in CD27+IgD+ unswitched memory B cell profiles

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1. Contribution

As the first author, my contributions to this research paper encompassed the study design, planning, execution, and analysis of all experiments, along with the article writing. For the statistical analysis and figure generation I was supported by Eduardo Rosales and Franck Ngangom.

2. Background and introduction

The pathogenesis of RA is complex, involving a combination of genetic predispositions, environmental factors, and dysregulated immune responses as elaborated in chapter IV. Altered profiles of various immune cells are central to the pathophysiology of RA [8]. Understanding the dynamics of the immune system is crucial for developing effective therapies and diagnostic tools[136]. Traditionally, immunophenotyping has relied on flow cytometry to analyze the composition and functional states of immune cells in peripheral blood and affected tissues. However, this technique is limited to measure a small number of parameters simultaneously. Cytometry by Time-of-Flight (CyTOF), also known as mass cytometry, represents a significant improvement in this field as it allows the simultaneous measurement of over 30 markers at the single-cell level, providing a more detailed view of immune cell populations and their states[137,138]. Previously reported key players in the auto-immune disease RA include T cells, B cells, macrophages, and dendritic cells, each contributing to the chronic inflammatory environment characteristic of RA[8]. T cells produce pro-inflammatory cytokines like IL-17 and IL-21. B cells generate autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), which are central to the disease's pathology in patients with seropositive RA[23]. Monocytes and macrophages also play crucial roles by producing inflammatory cytokines such as TNF-α and IL-1β, driving synovial inflammation and joint destruction[139]. Dendritic cells, including myeloid (mDCs) and plasmacytoid (pDCs) subtypes, are involved in antigen presentation and cytokine production, further modulating the immune response in RA[140]. Due to the versatile function of the different immune cells and their interplay in RA there remains a need for comprehensive and high-resolution profiling of immune cell subsets to fully elucidate their roles and interactions in RA.

The Maxpar Direct Immune Profiling Assay (MDIPA) is a standardized panel of metal-conjugated antibodies designed for CyTOF. This panel includes markers for a broad range of immune cell types, including T cells, B cells, NK cells, monocytes, and dendritic cells, enabling comprehensive immune profiling. MDIPA staining is performed on whole blood samples, which simplifies the workflow and reduces sample handling variability, ensuring consistency and reproducibility in data collection[141].

By comparing MDIPA stained whole blood samples run on CyTOF from RA patients and healthy controls recruited for the ExpoBiome study, the aim was to reveal differences in cell populations and states that may be associated with disease presence and severity.

3. Manuscript

Immunophenotyping of patients with rheumatoid arthritis reveals difference in CD27^{+}IgD^{+} unswitched memory B cell profiles

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Abstract

Objectives

Over the past decades, the prevalence of non-communicable diseases has surged significantly, including the systemic autoimmune disorder rheumatoid arthritis (RA). Despite extensive research and advancement of RA therapy, effective prevention strategies or cures remain elusive and the mechanisms underlying RA pathogenesis unclear.

It is crucial to gain deeper insights into RA pathophysiology. The objective of this study is to provide a comprehensive immunophenotyping of patients with RA.

Methods

We generated and analysed deep immunophenotyping data from 60 patients with RA and 60 healthy controls (HC). Whole blood samples were stained with extracellular markers, and intracellular antibodies, and analysed for 32 different cell markers using mass cytometry by time of flight. The acquired data was analysed by both manual and automatic unsupervised tools and subsequently complemented with anthropometric data and clinical-laboratory parameters.

Results

We observed a significant disparity in immune cell profiles between patients with RA and HC, notably a reduced frequency of $CD27^+IgD^+$ unswitched memory B ($_{m}$B) cells in patients with RA (*P*-value < 0.01), with the disease RA being the primary and only significant factor explaining up to 17.9% of the variance of these cells.

Conclusion

Our results reveal, for the first time, that a reduced frequency of unswitched $_{m}$B cells in patients with RA is the only significant abnormality distinguishing patients with RA from HC in a complex immunophenotyping panel of 72 different cell populations. This provides an important information to further understand differential responses to various interventions and possibly help to design novel therapeutic interventions.

Keywords: Rheumatoid arthritis, CyTOF, immunology, autoimmunity, immunophenotyping, $IgD^{+}CD27^{+}$ unswitched memory B cells

Introduction

Western societies are burdened by an increased incidence of non-communicable diseases resulting in declining overall health and complications later in life [1, 2]. RA is a non-communicable disease, affecting about 1% of the worldwide population, with women being at a threefold higher risk compared to men [3]. RA is a systemic autoimmune disease, primarily affecting the synovial lining of the joints [4]. The chronic systemic inflammation may also involve the lungs, vasculature, and bones. Clinically, this polyarthritis manifests primarily in joint pain and untreated in mutilating joint destruction, eventually severely impacting the quality of life of patients. The pathogenesis of RA involves a complex interaction of immune cells, including T cells, B cells, dendritic cells, natural killer cells and monocytes[5, 6]. $CD4^+$ T cells have been described as pivotal in RA, interacting extensively with B cells, dendritic cells and fibroblast-like synoviocytes[5, 6]. B cells contribute to RA by producing autoantibodies such as rheumatoid factor and anti-citrullinated protein antibodies, which are detectable in patients with seropositive RA, and present antigens to T cells [7]. Most mature B cells can be classified into four different subtypes based on the IgD and CD27 surface markers (Table 1). $CD27^+$ cells are larger and possess immunoglobulin producing capabilities [8]. It has been suggested that unswitched $_m$B cells are innate-like B cells (ILB) or circulating marginal zone B cells which develop independently of the germinal center response [9, 10]. Metabolic reprogramming leads to the differentiation of human unswitched memory B cells into plasmablasts or $CD27^-IgD^-$ memory B cells [9]. Despite undergoing somatic hypermutation, unswitched $_m$B cells do not undergo class switch recombination. A distinctive feature of this subset is their role in the first line of defence. They produce IgM in response to pathogens, which has also been hypothesized to have a protective role in autoimmune diseases [9, 10]. RA is a highly heterogenous disease and the immunophenotype goes far beyond the simple classification of seropositive or seronegative RA

[11]. Patients have been reported to respond differently to various treatment options, show different progression rates, distinct comorbidities, and overall differing phenotypes [12]. Likely, the clinical features depend on the heterogenous immunophenotype in a non-linear fashion. Furthermore, several cell subsets are known to response in an environment-dependent way, including dendritic cellss, $\gamma\delta$ T cells, B cells and natural killer cells [13, 14]. To date, the disease is treated with anti-inflammatory drugs for the acute flares and with long-acting immune suppressing or modulating therapies to influence the course of the disease. Both a sustainable and efficient prevention as well as a cure are currently lacking [15]. Accumulating evidence suggests a pivotal role for environmental factors, including nutrition, physical activity, lifestyle interventions like caloric restriction, and the gut microbiome, in RA pathophysiology [16]. Yet, elucidating the immunophenotypic landscape of this chronic inflammatory disorder and the immunological pathways influenced by therapeutic interventions remain key for advancing our understanding of RA pathogenesis and treatment strategies.

To deeper understand the immunophenotypic landscape of RA, we characterized the blood of patients with RA using CyTOF, employing a high dimensional approach to detect marker combinations and cluster generation taking into consideration clinical and laboratory features [4]. We aimed to compare the obtained profile to healthy controls, displaying distinct immunological differences to enable better understanding of pathophysiology and open opportunities for individual, targeted treatments [17].

Results

Clinical cohorts

A final number of 99 samples was included in the analysis, including 47 HC and 52 patients with RA. We omitted 21 samples due to missing values, either immunological or clinical, resulting in n = 52 patients with RA and n = 47 HCs. The clinical and anthropometrical characteristics are

139 summarized in (Table). Overall, the CDAI was high for the patients with RA, signalling a state of
140 active disease and acute flares [18] (Table 2). We observed statistically significant differences
141 between the two cohorts: for patients with RA we observed a lower number of hours of sleep,
142 lower WHR, reduced frequency of walking for longer than 10 minutes and lower creatinine
143 levels compared to HC (Table).
144
145 *High dimensional comparison of immune cell profiling in patients with RA and HC*
146 We compared immune cell frequencies between patients with RA and HCs. Most cell types
147 studied did not exhibit significant differences after correcting for multiple comparisons.
148 However, we found significantly lower cell frequencies of unswitched $_{m}$B cells in patients with
149 RA compared to HC in the supervised, hierarchical analysis in both total CD45^{+} cells (*P*-value =
150 0.0032) and total B cells (*P*-value = 0.0098) (Figure 1; Figure 2). Although the $\gamma\delta$ T cells also
151 trended to be lower in patients with RA, this difference did not reach statistical significance
152 after adjustment for multiple comparisons (*P*-value > 0.05 in FDR) [19, 20]. For the automated
153 unsupervised analysis performed by the CellEngine software, the best distribution and
154 differentiation of the cells displayed by the expression of their respective markers in form of a
155 heatmap could be observed with a total of 100 clusters (10x10). To avoid dispersion of
156 neutrophil populations into numerous clusters, and thus conceal less frequent cell subsets, a
157 more targeted approach was applied to better define the cluster identities. This was done by
158 applying the 10x10 clustering to a cell subset, excluding neutrophils (Supplementary figure 5).
159 No significant differences were observed between the two groups in the unsupervised analysis.
160 Together, the supervised immunophenotyping demonstrated a significantly lower number of
161 unswitched $_{m}$B cells in patients with RA compared to HC.
162

Integration of Clinical data and treatment

Potential confounding factors did not significantly correlate, confirming their suitability for our models (Supplementary figure 6). Two linear regression models were constructed to assess the impact of various factors on unswitched $_{m}$B cell frequencies in patients with RA and HC. The first model, including both RA patients and HC, revealed that RA was the only significant predictor of unswitched $_{m}$B cell frequency ($CD45^{+}$ parent population: $\beta = -0.752$, $p < 0.01$; B cell parent population: $\beta = -0.365$, $p < 0.05$), independent of other covariates (WHR, creatinine, sleep, walking frequency). This model explained 17.9% of the variance in unswitched $_{m}$B cell levels in the $CD45^{+}$ cell population and 16.9% in the total B cell population ($p < 0.01$) (Supplementary figure 7). The second linear regression model, focusing on potential confounders within the RA group alone, indicated that none of the variables, including disease duration and medication type, significantly impacted unswitched $_{m}$B cell frequency. This model also exhibited a low adjusted R-squared value, suggesting that the included confounders did not contribute substantially to explain the variation in cell measurements. We also corrected for the impact of medication on the unswitched $_{m}$B cell frequency. We differentiated between five different treatment groups: conventional DMARDs, biologic and targeted synthetic DMARDs, glucocorticoids, combined treatment and no specific RA medication (Supplementary figure 8). The conventional DMARDs consisted mostly of methotrexate, but also included Leflunomide and Sulfasalazine. The treatments in the biologic and targeted DMARD group were Adalimumab, Sarilumab, Golimumab, Etanercept, Tocilizumab and Baricitinib. The glucocorticoid medication was exclusively Prednisolone. We detected no significant difference in unswitched $_{m}$B cells between the different medication treatment groups (Kruskal-Wallis test by ranks) [21] (Figure 3). Hence, the significantly lower number of unswitched $_{m}$B cells in patients

with RA was not impacted by any confounder or medication and might be a key characteristic of the autoimmune disease.

Discussion

Regarding the phenotypic characterization, we found several differences, namely creatinine, duration of sleep, walking frequency and WHR, in clinical and anthropometrical characteristics between patients with RA and HCs, some of which might be explained by the nature of the disease [22-24]. The slightly reduced amount of sleep recorded for patients with RA might be explained by typical RA symptoms such as joint pain and stiffness, as well as side effects of medication or comorbidities, including anxiety and depression and a higher prevalence of sleep-related breathing disorders, leading to sleep disturbance [25]. Also, although walking is classified as a feasible, safe and beneficial intervention for patients with RA, these patients spent generally less time walking than the HCs, which might be associated with joint pain and other RA symptoms like fatigue [24]. Creatinine levels were significantly lower in patients with RA, which could be due to an increased risk for sarcopenia in RA or side effects of RA specific medication [26, 27]. The observed lower WHR in patients with RA is atypical as the chronic inflammation in combination with medication and reduced physical activity often leads to an increased WHR compared to HC [28]. We expected to observe a distinct immunological profile in patients with RA compared to controls and were able to confirm this in our analysis. Despite the ongoing medical treatment, we found a significantly lower number of unswitched $_{m}$B cells in patients with RA compared to HCs. Additionally, $\gamma\delta$ T cells were lower in patients with RA. However, after correction for multiple testing the difference was no longer significant. Previous studies have reported elevated B cell numbers in RA, which is a phenomenon generally anticipated in autoimmune diseases [29]. Treatment strategies therefore often focus on B cell

depletion in such scenarios due to the pivotal role of these cells in antigen presentation, cytokine and antibody production [30]. Conversely, for the specific unswitched $_{m}$B cell subset, lower levels have been previously reported for patients with systemic lupus erythematosus, patients with Sjogren's syndrome as well as for patients with RA compared to control subjects [9, 31]. Although unswitched $_{m}$B cells carry higher potentials of inflammatory response than CD27^{-} B cells, they have been reported to negatively correlate with disease activity [10]. Also, it was found that in B-cell depletion therapy-naïve patients with RA, the frequency of unswitched $_{m}$B cells correlated inversely with levels of serum B cell activation [32]. In addition, differences in serum immunophenotypes of B cells in autoantibody-positive and -negative RA have been found and add to the complexity of the immunophenotyping of patients with RA [33]. Their function was found to be impaired, including a decreased IgM production [10]. As IgM antibodies have been recognized to play protective roles in autoimmune diseases, one possible key role of unswitched $_{m}$B cells in RA could be linked to reduced IgM producing capacities [10]. Also, the possible migration of the cells to the inflamed synovium or the differentiation into other subsets might explain the reduced levels of these unswitched $_{m}$B cells found in patients with RA [9]. Although the exact role of the unswitched $_{m}$B cell subset in RA is not clear yet, a major role is suggested, with the disease RA being the primary and only significant factor explaining up to 17.9% of the variance of unswitched $_{m}$B cells in our model. The previously reported findings and our results emphasize the importance of better understanding the role of unswitched $_{m}$B in autoimmunity and optimizing treatment and prevention in patients with RA. $\gamma\delta$ T cells have also been previously reported to be lower in patients with RA in 1999 [34]. This finding has then been both confirmed and challenged in the past years and could not be confirmed in this study [34, 35]. Several cell-subsets have been reported to adapt their response according to the environment to an either pro-inflammatory or tolerogenic response [36]. This is

234 also the case for specific subsets of $\gamma\delta$ T cells that have been suggested to play an important
235 role in inflammatory response [35]. $\gamma\delta$ T cells can exert different effector phenotypes, amongst
236 others cytotoxicity, cytokine production, and immunoregulatory functions [13, 37]. This
237 environment-dependent response of some immune cell subsets is a crucial aspect that must
238 be considered for future research, prevention, and treatment therapies. Both the
239 heterogeneity of RA and the adaptive cell-response might explain why, besides the difference
240 in unswitched $_{m}$B cells, no obviously distinct pattern could be detected comparing patients with
241 RA to HCs. Although reduced numbers of unswitched $_{m}$B cells in patients with RA have been
242 reported in 2009 and 2018 by using specific staining (e.g. CD19, CD27 and IgD) on isolated
243 PBMCs or whole blood by using FACS or FlowCytometry respectively, we show for the first time,
244 that a reduced frequency of unswitched $_{m}$B cells is the only significant difference distinguishing
245 the immunophenotype from patients with RA from HC in a complex immunophenotyping panel
246 of 72 different cell populations[10, 31].
247 The findings of our study highlight the complex and dynamic role of the immune system in the
248 chronic, systemic, autoimmune disease RA. Understanding the underlying mechanisms and
249 consequences of the reduced number of unswitched $_{m}$B cells in patients with RA could provide
250 valuable insights into RA pathogenesis and lead to the development of more targeted and
251 effective therapeutic strategies. Further research is necessary to elucidate the precise role and
252 underlying mechanisms of these cells and their potential as biomarkers or therapeutic targets
253 in RA.
254

255 ## METHODS

256 *Sample collection*

The samples for the CyTOF analysis were collected as part of the ExpoBiome study [4]. The patients were either diagnosed with RA or classified as healthy controls and included according to the exclusion and inclusion criteria[4]. Healthy controls were without any evidence of active known or treated RA. The cohorts were matched for age and gender. Ethical approval was given by the Ethics Committee of Charité-Universitätsmedizin Berlin (EA1/204/19), the Ethics Committee of the State Medical Association of Hesse (2021-2230-zvBO) and the Ethics Review Panel (ERP) of the University of Luxembourg (ERP 21-001 A ExpoBiome). The study was registered in Clinicaltrials (https://clinicaltrials.gov/ct2/show/NCT04847011).

Sample processing and extracellular staining for CyTOF

Immediately after blood collection in heparin tubes, the whole blood samples were stained with the MaxPar Direct Immune Profiling Assay (MDIPA, Standard Biotools, CA, USA) and stabilized with Prot1 stabilizer (SmartTube Inc. San Carlos, CA, USA) according to a previously validated workflow [38] (Supplementary table 1). The samples were stored at –80°C until further processing.

Intracellular staining and acquisition for CyTOF

Before additional intracellular staining with the in-house conjugated antibodies, all antibodies have been titrated (Supplementary table 2). The subsequent sample preparation was done according to the manufacturer's protocol (Standard Biotools, CA, USA). The whole blood samples were thawed in a 12°C water bath before a thaw lyse buffer, prepared from a 1:2000 dilution of 1000X concentrate (SmartTube Inc. San Carlos, CA, USA) and MilliQ water was added. The samples were incubated for 10 min. After centrifugation, MaxPar Cell Staining Buffer (CSB, 201068, Standard Biotools, CA, USA) was added to the sample. The samples were

281 treated with the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (00-5523-00,
282 invitrogen, MA, USA) and then stained with the optimal concentration of in-house conjugated
283 antibodies before a 30 min incubation at 4°C. After several washing steps, a freshly prepared
284 1.6% formaldehyde solution (Pierce, 16% Formaldehyde, 289006, Thermo Fisher Scientific) was
285 added to the samples. A multiplexing strategy based on the use of the Cell-ID™ 20-Plex Pd
286 Barcoding Kit (Cell-ID, 201060, Standard BioTools, CA, USA) was applied which enabled the
287 analysis of up to 20 samples per experiment (Supplementary figure 1). A DNA Ir-intercalator
288 solution (201192A, Standard Biotools, CA, USA) was prepared. After dissolving the barcodes in
289 100 µL of a diluted Ir-solution and adding them to the samples, the cells were resuspended in
290 the remaining DNA Ir-intercalator solution to a final concentration of 50 nM Ir-Intercalator per
291 3 x 10^6 cells and incubated overnight at 4°C. Prior to the acquisition, the barcoded cells were
292 pooled together and underwent further washing steps with Maxpar Cell Acquisition PLUS (CAS
293 PLUS, 201244, Standard Biotools, CA, USA). 10% calibration beads (Maxpar Four Elements EQ
294 Beads, 201078, Standard Biotools, CA, USA) were added to the sample before the acquisition
295 with a Helios mass cytometer (Standard BioTools, CA, USA). To avoid batch effects and monitor
296 technical variation, the sample acquisition was randomized, and a reference sample was
297 included in each CyTOF run (Supplementary figure 2).
298
299 *Hierarchical gating and unsupervised analysis of CyTOF data*
300 After the CyTOF acquisition, the Flow Cytometry Standard (FCS) data files were debarcoded
301 using an integrated debarcoder tool in the CyTOF software 7.1 according to the manually set
302 Minimum Barcode Separation parameter. The newly generated debarcoded FCS files were
303 imported into FlowJo™ v10.9 software (BD Life Sciences, Ashland, USA) and a gating strategy
304 was established to identify and characterize 72 relevant immune cell populations

(Supplementary figure 3). Information on the number of each cell population and their respective parental frequencies (%) was exported for further analysis of the hierarchical and supervised data. Using Tableau Prep Builder v2021.4 (Tableau Software, LLC, Washington, USA), a pipeline to prepare and organize the exported data was generated [39]. Additional sample information was added to the pipeline and the consequent database used in Tableau Desktop v2023.2 (Tableau Software, LLC, Washington, USA) included additional metrics.

In addition to the hierarchical gating analysis, we performed an automated, unsupervised analysis clustering cell populations based on similar protein marker expression profiles [40]. This analysis was performed using the CellEngine software (CellCarta, Montreal, Canada). Preliminary tests using FCS with data on $CD45^+$ cells were run to evaluate different FlowSOM parameters, notably, the numbers of final clusters and the number of consensus clusters. We tested different conditions, including 10x10 and 12x12 final clusters with 12 or 24 consensus clusters. The expression of the different markers visualized as heatmaps was used to identify the cell populations representing the different clusters in an unbiased way. After establishing the identity of the major cell populations with the newly generated heat map, the cluster ID information was imported into the Tableau Prep pipeline.

Statistical analysis of hierarchical and unsupervised analysis of CyTOF data

The calculated metrics and resulting data were imported into Qlucore Omics Explorer 3.8.1 (Qlucore, Sweden) to assess statistical differences between clusters or cell populations and cohorts by calculating *P*-values and *Q*-values, using different parent populations as reference for the hierarchical dataset ($CD45^+$ Live, B-cells, $CD3^-CD19^-$, $CD4^+$, $CD8^+$, MAIT). The Mann-Whitney U test was applied to the hierarchical and unsupervised dataset.

Clinical data integration and statistical analysis

Clinical data collected in REDCap was integrated into the data acquired by CyTOF using R (R 4.3.2) and R studio (2023.09.1+494) (Posit PBC, Boston, USA). The baseline characteristics of patients with RA and HC have been compared using a *Mann-Whitney U-test.* Possible confounders were selected, including age, gender, diet, waist hip ratio (WHR), body mass index (BMI), wellbeing score (WHO-5), hours of sleep and time spent walking, albumin, creatinine, insulin, C-reactive protein (CRP), cholesterol, glucose and thyroid stimulating hormone (TSH) levels. The selected integrated factors were tested for correlation to ensure they were independent. To test these possible confounding factors, a linear regression model with the disease RA as predictor was built. Variables were visually inspected using histograms, tested for normality by applying the Shapiro-Wilk-test, and log-transformed when necessary to meet linear regression assumptions. A second linear regression model was built to define further explanatory factors for acquired cell counts in patients with RA. This model looked at specific RA predictors including the duration of disease, clinical disease activity score (CDAI), rheumatoid factor and CRP. The *P*-values were adjusted according to the Benjamini-Hochberg false-discovery rate method for each model to correct for multiple testing [19, 20]. A P-value < 0.05 was regarded as statistically significant. In addition, as most of the patients with RA were not treatment-naïve at the time of the blood sampling a Kruskal Wallis test was run to account for different treatment groups (Supplementary figure 4) [21].

Limitations of the study

The limitations of the study include a reduced sample size, as the sample size of initially n = 120 patients was reduced to n = 99 patients due to missing clinical values. The analysis in patients

with RA was done on n = 52 patients, which is further limited by different medical treatments of the patients. As the study was designed to compare the immunophenotype of patients with RA to HCs, this study lacks the statistical power (power = 0.13) to deeply analyse effects of medication, heterogenous RA pathophysiology and environment-dependent cell response. However, this analysis was not the aim of this study and should be further investigated in future clinical trials.

FUNDING STATEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Study design and protocol were done by Bérénice Hansen, Cédric C. Laczny, Jochen G. Schneider, Paul Wilmes; the interventional concept was drawn by Etienne Hanslian, Daniela A

Koppold Andreas Michalsen, Anika Rajput Khokhar, Brit Mollenhauer, Sebastian Schade, Nico Steckhan, Jochen G. Schneider, Paul Wilmes; the clinical trial was designed and conducted by Etienne Hanslian, Daniela A Koppold, Michael Jeitler, Andreas Michalsen, Anika Rajput Khokhar, Brit Mollenhauer, Sebastian Schade; the procured funding was provided by Paul Wilmes; the statistical analysis was done by Bérénice Hansen, Eduardo Rosales Jubal , Franck Ngangom, Viacheslav Petrov, Rajesh Rawal, Michael Vaillant; sample size calculation were defined by Cédric C. Laczny, Jochen G. Schneider, Paul Wilmes, Kirsten Roomp; the initial draft of the manuscript and coordination of the editing process was performed by Bérénice Hansen; the sample protocol preparation has been done by Bérénice Hansen, Maira Konstantinou, Dominique Revets; the planning of the data analysis was done by Cédric C. Laczny, Jochen G. Schneider, Paul Wilmes, Kirsten Roomp, , Raul Da Costa, Fanny Hedin, Antonio Cosma; the CyTOF experiments and data analysis were designed and performed by Bérénice Hansen, Raul Da Costa, Dominique Revets, Fanny Hedin, Maira Konstantinou , Antonio Cosma; all authors contributed equally with edits, comments and feedback, read and approved the final manuscript.

DATA Availability Statement

All relevant patient data used in this study can be requested by contacting the corresponding authors.

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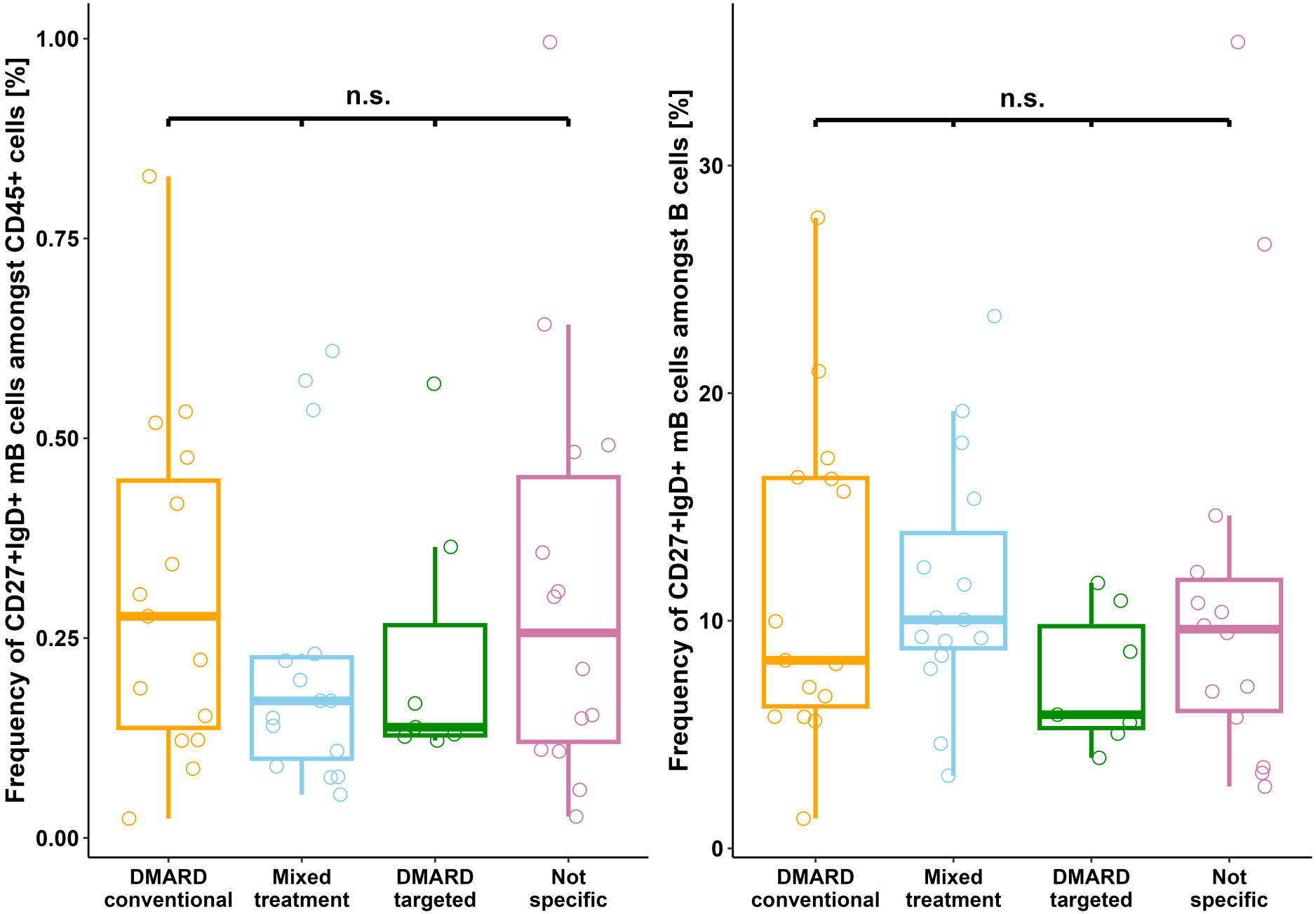
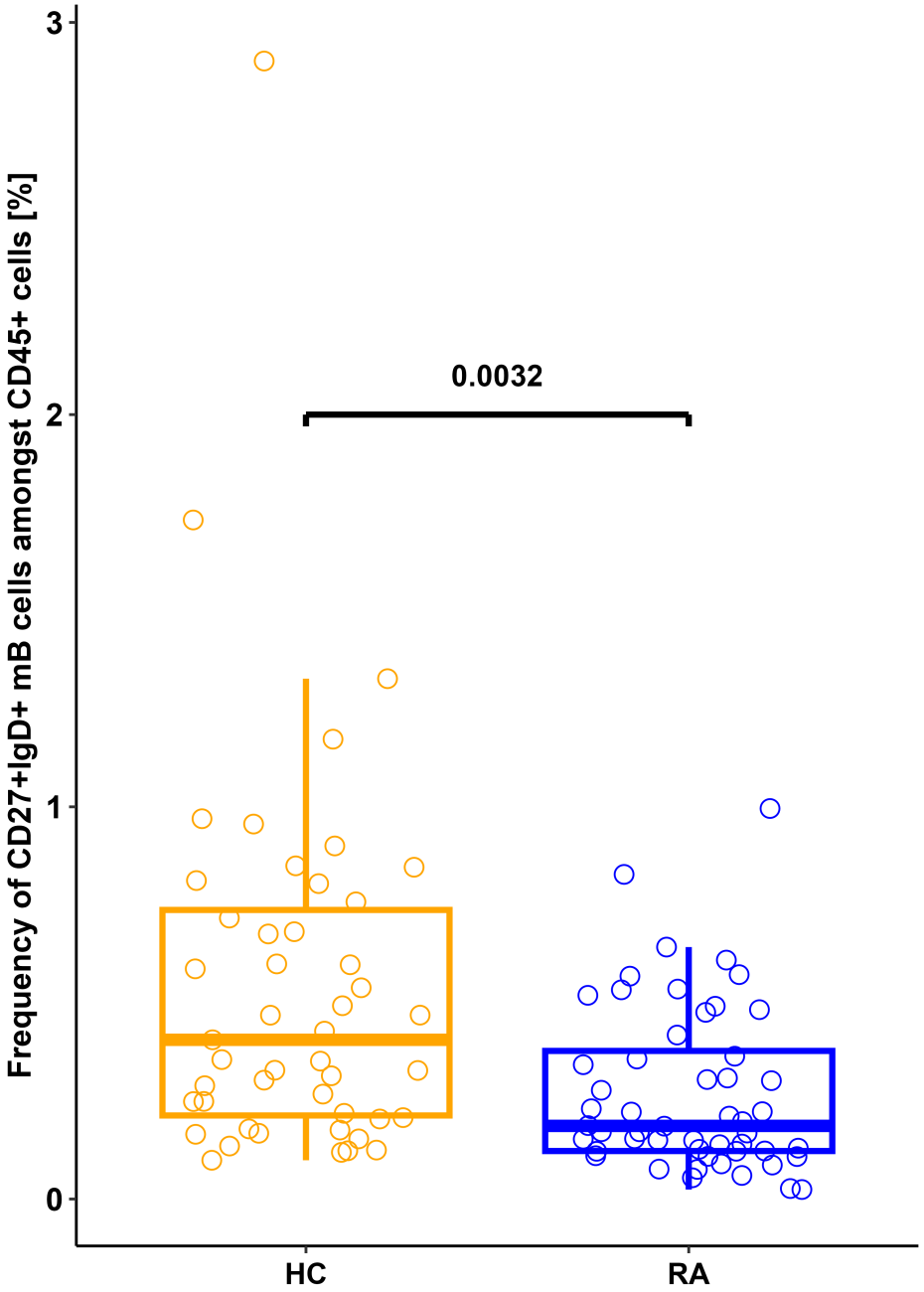
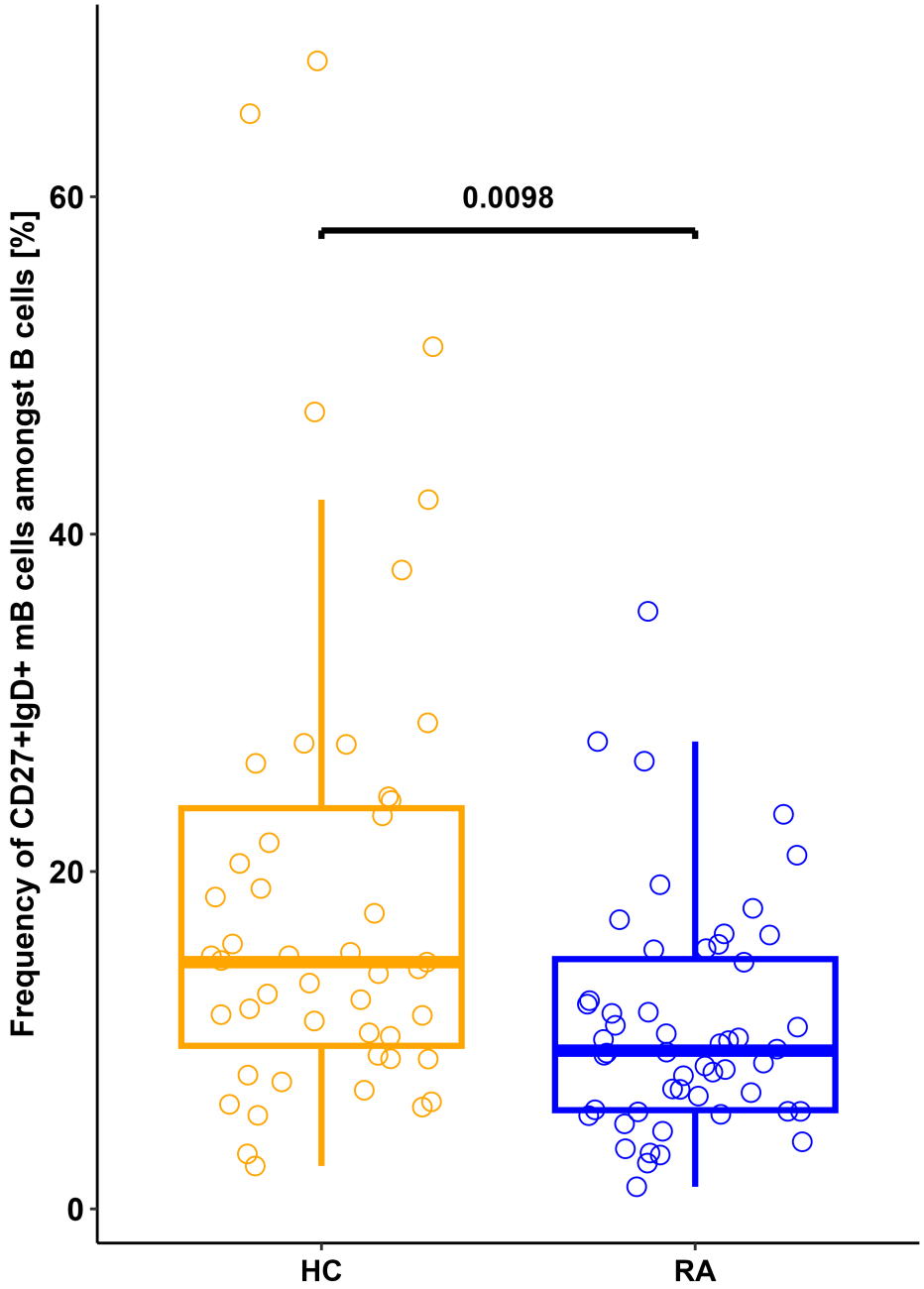
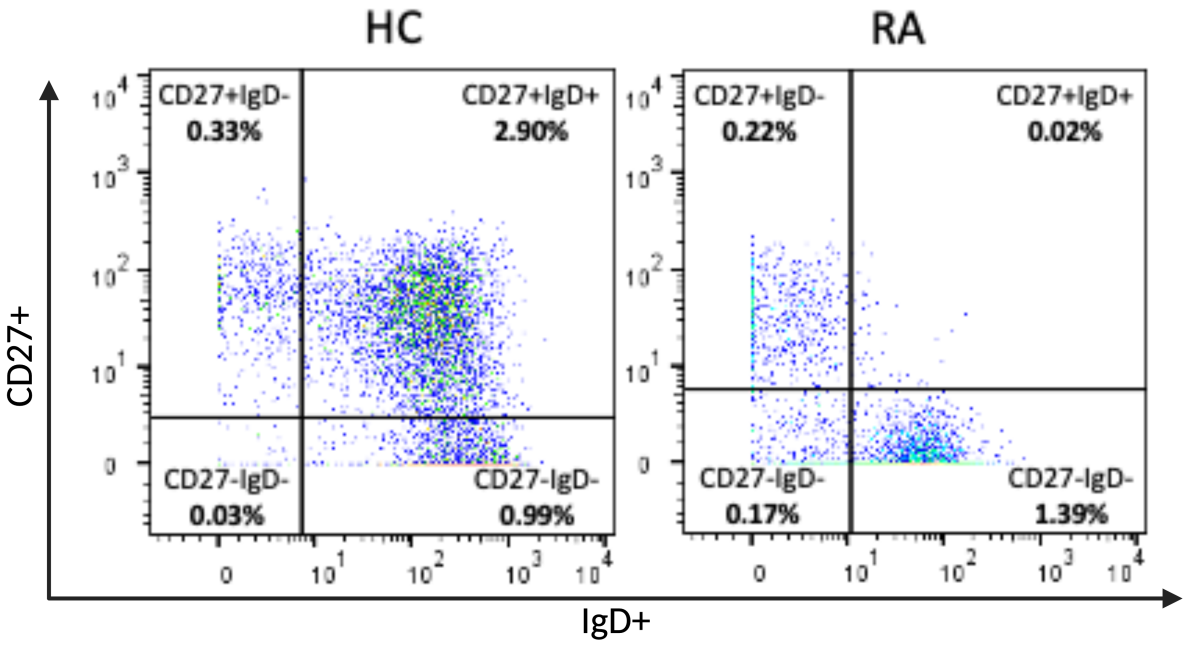

Frequency of CD27+IgD+ mB cells amongst CD45+ cells [%]
1.00
0.75
0.50
0.25
0.00
n.s.
DMARD conventional
Mixed treatment
DMARD targeted
Not specific
Frequency of CD27+IgD+ mB cells amongst B cells [%]
30
20
10
0
n.s.
DMARD conventional
Mixed treatment
DMARD targeted
Not specific


Frequency of CD27+IgD+ mB cells amongst CD45+ cells [%]
3
2
1
0
0.0032
HC
RA


Frequency of CD27+IgD+ mB cells amongst B cells [%]
60
40
20
0
0.0098
HC
RA


HC
RA
CD27+IgD-
0.33%
CD27+IgD+
2.90%
CD27-IgD-
0.03%
CD27-IgD-
0.99%
CD27+IgD-
0.22%
CD27+IgD+
0.02%
CD27-IgD-
0.17%
CD27-IgD-
1.39%
CD27+
IgD+

501 Tables

502 Table 1: Memory B cell subtypes based on their cell surface expression of markers CD27 and
503 IgD.

Memory B cell subtype	CD27	IgD
Unswitched	+	+
switched	+	–
Naïve	–	+
Double negative	–	–

504

505 **Table 2:** Baseline characteristics of patients with RA (n = 52) and healthy controls (n = 47). **P*-
506 value < 0.05, ***P*-value < 0.01 (RA vs HC); y = years; BMI = body mass index; WHR = waist-hip-
507 ratio; WHO-5 = wellbeing score; CRP = C-reactive protein

	Rheumatoid Arthritis (n = 52)		**Control Group (n = 47)**		***P*-value**
Variable	Median	IQR	Median	IQR	
Age[y]	55.23	12.56	57,23	14.93	0.5656
Female [%]	88,3		72,1		0.0648
BMI	23,94	5.83	24,21	5.76	0.5752
WHR**	0,81	0,07	0,88	0,1	0.0004
WHO-5	13.5	8	17	9.5	0.0569
Sleep (h)*	6	2	7	2	0.0118
Walking for 10 min**	2	1	2	0	0.0055

Diet [% omnivore]	53.1		61		0.3029
Albumin [g*L^{-1}]	42.5	4.075	43.1	2.6	0.0679
Creatinine [µmol*L^{-1}]**	58.5	11.88	66.7	11.85	0.0000
hs-CRP [mg*L^{-1}]	1.7	2.55	1.04	1.085	0.6948
Insulin [mU/L^{-1}]	6.4	3.45	5.5	3.1	0.1750
TSH basal [mU*L^{-1}]	0.99	0.68	1.11	0.85	0.1997
Cholesterol [mmol*L^{-1}]	5.83	1.35	5.59	1.745	0.7792
Glucose [mmol*L^{-1}]	5.035	0.7525	4.98	1.0775	0.6513

*P-value < 0.05 (RA vs HC), **P-value < 0.01 (RA vs HC)

Table 2: Specific baseline characteristics of patients with RA (n = 52). CDAI = clinical disease activity index; y = years

Variable	Median	IQR
CDAI	55	82.75
Rheumatoid factor [IU*mL^{-1}]	18.3	37.3
Disease duration [y]	7.25	13.76

Figure Legends

Figure 1: Representative CD27 IgD dot plots from a healthy control (HC) and a patient with rheumatoid arthritis (RA) indicating the distribution and percentages of the following memory B cell subsets in the total $CD45^+$ cell population: $CD27^+IgD^+$ (unswitched); $CD27^+IgD^-$ (switched); $CD27^-IgD^+$ (naïve); $CD27^-IgD^-$ (double negative).

Figure 2: Differences in cell expression in unswitched memory B ($_mB$) cells in patients with rheumatoid arthritis (RA) compared to healthy controls (HC). The left plot illustrates the frequency of unswitched $_mB$ cells as a proportion of the overall population of $CD45^+$ cells, whereas the right plot depicts the frequency of unswitched $_mB$ cells relative to the total B cell population.

Figure 3: Impact of different treatments on cell expression in unswitched memory B ($_mB$) cells in patients with rheumatoid arthritis (RA) compared to healthy controls (HC), the left plot illustrates the frequency of unswitched $_mB$ cells as a proportion of the total B cell population, whereas the right plot depicts the frequency of unswitched $_mB$ cells relative to the overall population of $CD45^+$ cells, based on the hierarchical analysis. The patients were separated into four groups, based on their different treatments. No significant differences between the treatment groups were observed.

Supplementary description

Supplementary table 1: MDIPA extracellular antibodies used to stain whole blood samples
Supplementary table 2: Antibodies for intracellular staining of the extracellular stained and stabilized whole blood samples for CyTOF anaylsis, coupled in-house
Supplementary figure 1: Representation of the barcoding patterns
Supplementary figure 2: tSNE analysis of the reference sample pooled together
Supplementary figure 3: FlowJo Gating strategy

Supplementary figure 4: Flowchart of statistical analysis

Supplementary figure 5: Unsupervised clustering analysis

Supplementary figure 6: Spearman correlation for selected covariates

Supplementary figure 7: Impact of different factors on unswitched memory B (mB) cell frequency in patients with rheumatoid arthritis (RA) and healthy controls (HC).

Supplementary figure 8: Different treatment groups.

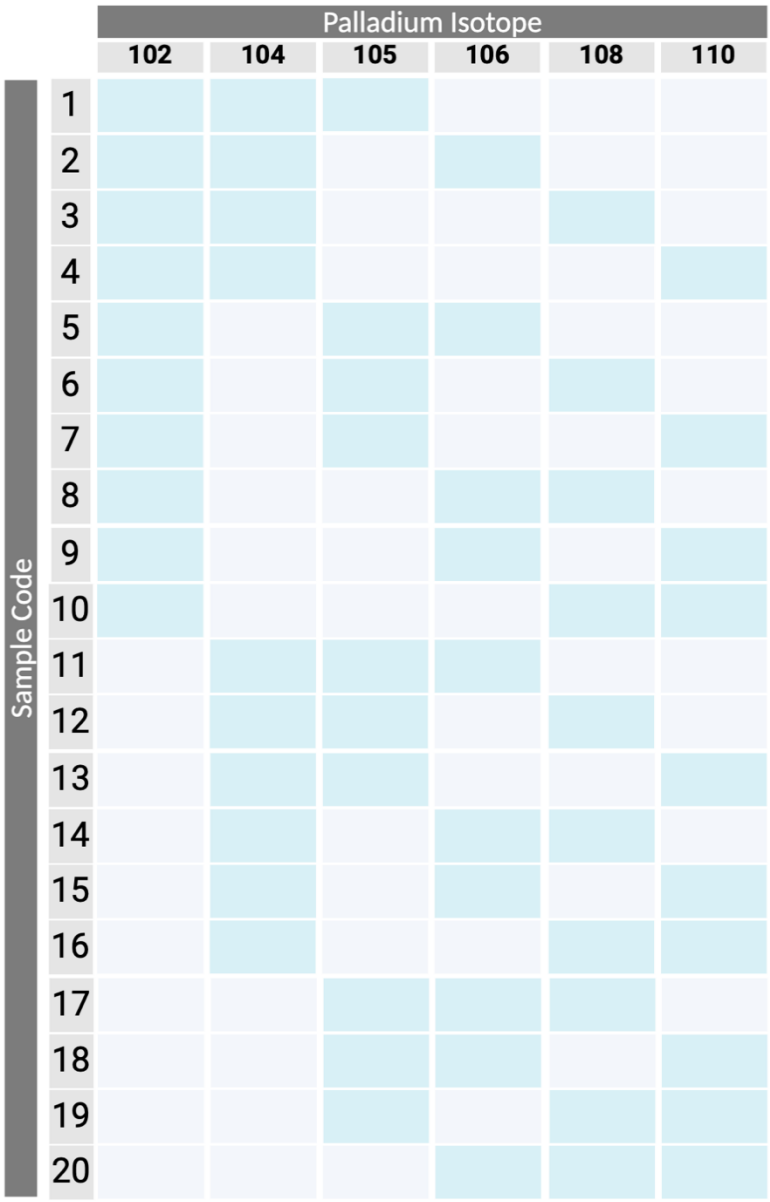
SUPPLEMENTARY TABLES AND FIGURES

Supplementary table 1: MDIPA extracellular antibodies used to stain whole blood samples

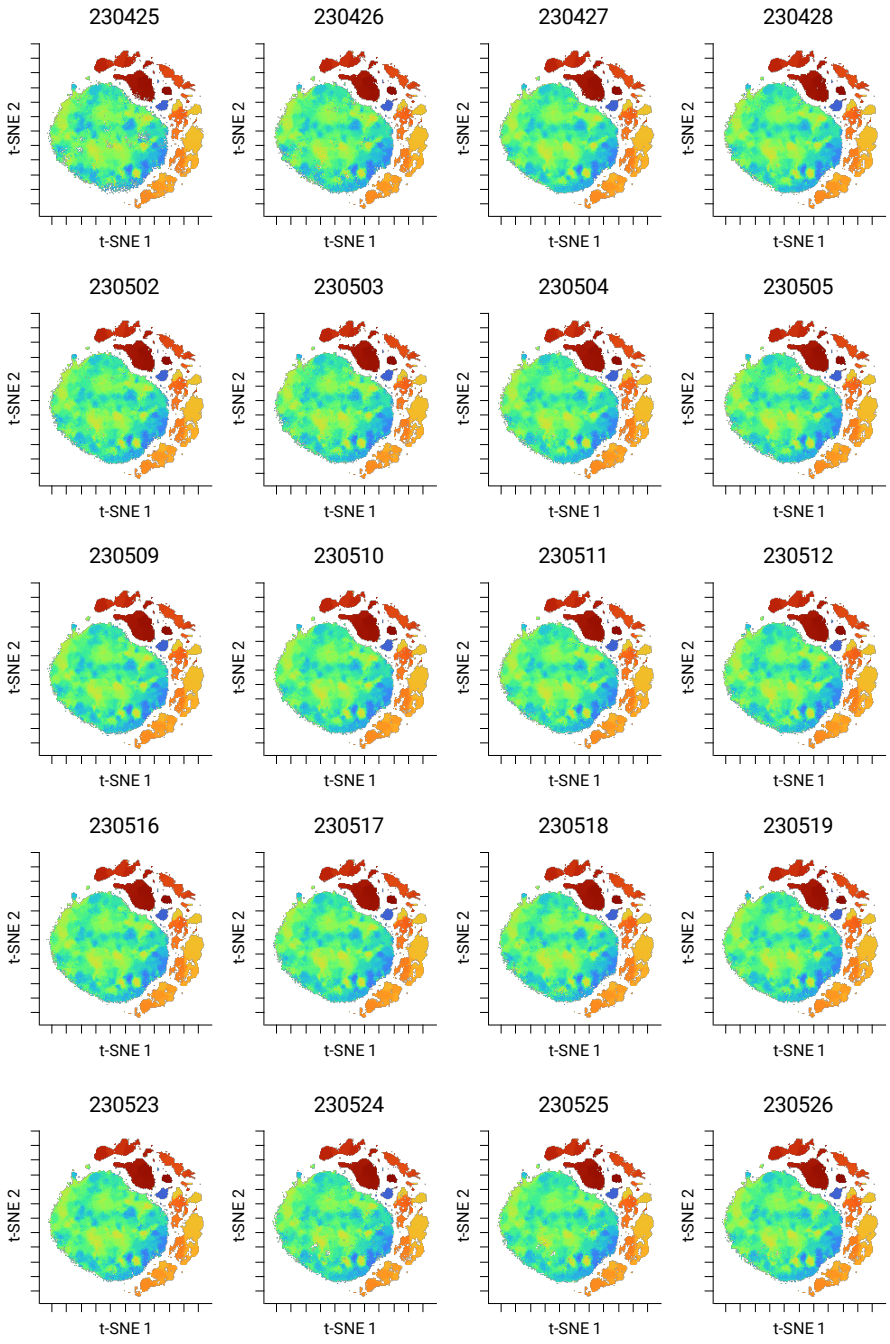
Metal Isotope	Antibody	Clone	Manufacturer	Catalogue#
89Y	CD45	HI30	Standard Biotools,	Part of MDIPA
103Rh	Live/Dead indicator		Standard Biotools	Part of MDIPA
141Pr	CD196 (CCR6)	G034E3	Standard Biotools	Part of MDIPA
143Nd	CD123	6H6	Standard Biotools	Part of MDIPA
144Nd	CD19	HIB19	Standard Biotools	Part of MDIPA
145Nd	CD4	RPA-T4	Standard Biotools	Part of MDIPA
146Nd	CD8a	RPA-T8	Standard Biotools	Part of MDIPA
147Sm	CD11c	Bu15	Standard Biotools	Part of MDIPA
148Nd	CD16	3G8	Standard Biotools	Part of MDIPA
149Sm	CD45RO	UCHL1	Standard Biotools	Part of MDIPA
150Nd	CD45RA	HI100	Standard Biotools	Part of MDIPA
151Eu	CD161	HP-3G10	Standard Biotools	Part of MDIPA
152Sm	CD194 (CCR4)	L291H4	Standard Biotools	Part of MDIPA
153Eu	CD25	BC96	Standard Biotools	Part of MDIPA
154Sm	CD27	O323	Standard Biotools	Part of MDIPA
155Gd	CD57	HCD57	Standard Biotools	Part of MDIPA
156Gd	CD183 (CXCR3)	G025H7	Standard Biotools	Part of MDIPA
158Gd	CD185 (CXCR5)	J252D4	Standard Biotools	Part of MDIPA
160Gd	CD28	CD28.2	Standard Biotools	Part of MDIPA
161Dy	CD38	HB-7	Standard Biotools	Part of MDIPA
163Dy	CD56 (NCAM)	NCAM16.2	Standard Biotools	Part of MDIPA
164Dy	TCRgd	B1	Standard Biotools	Part of MDIPA
166Er	CD294	BM16	Standard Biotools	Part of MDIPA
167Er	CD197 (CCR7)	G043H7	Standard Biotools	Part of MDIPA
168Er	CD14	63D3	Standard Biotools	Part of MDIPA
170Er	CD3	UCHT1	Standard Biotools	Part of MDIPA
171Yb	CD20	2H7	Standard Biotools	Part of MDIPA
172Yb	CD66b	G10F5	Standard Biotools	Part of MDIPA
173Yb	HLA-DR	LN3	Standard Biotools	Part of MDIPA
174Yb	IgD	IA6-2	Standard Biotools	Part of MDIPA
176Yb	CD127	A019D5	Standard Biotools	Part of MDIPA

Supplementary table 2: Antibodies for intracellular staining of the extracellular stained and stabilized whole blood samples for CyTOF anaylsis, coupled in-house

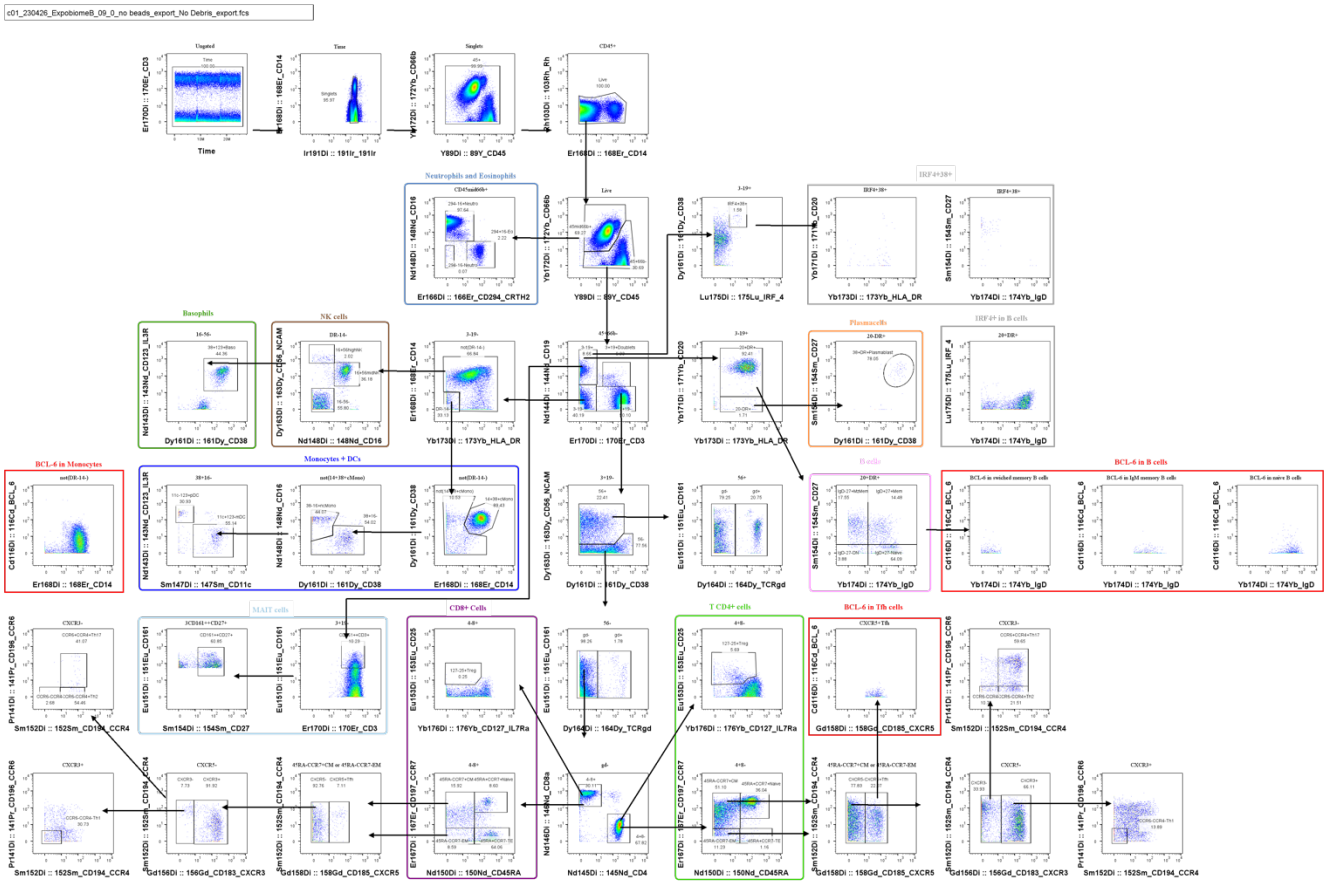
Metal Isotope	Antibody	Clone	Manufacturer	Catalogue#	uL/sample
116Cd	Bcl-6	REA373	Miltenyi	130-124-533	2
175Lu	IRF4	IRF4.3E4	Biolegend	646402	2



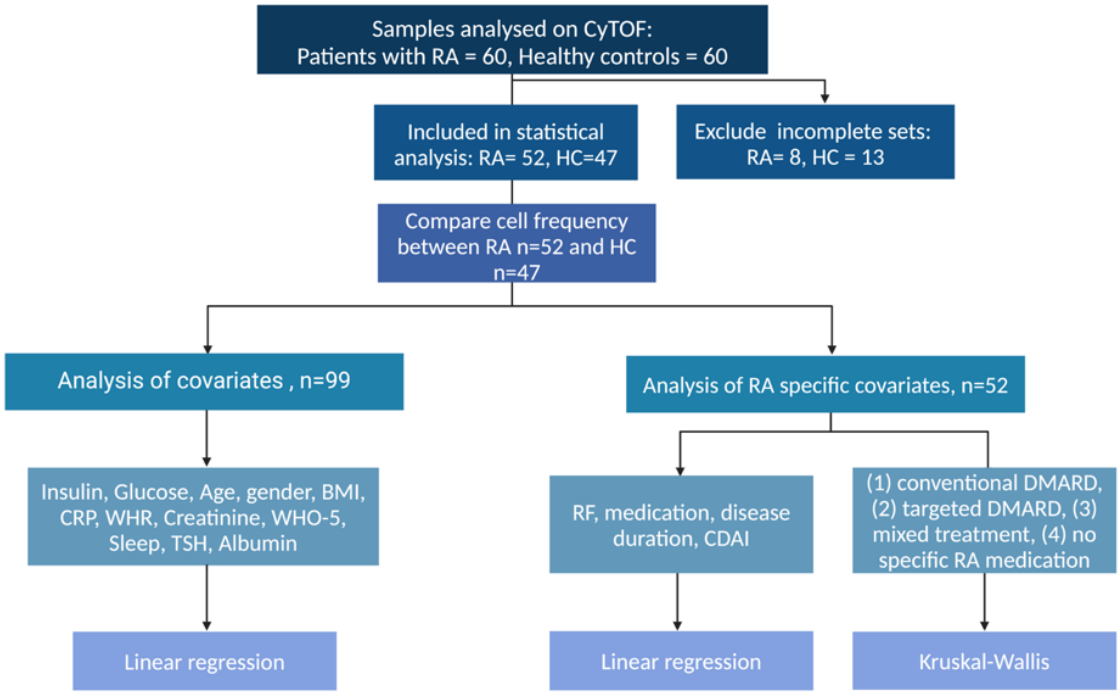
Supplementary figure 1: Representation of the barcoding patterns, each sample code consisting of a unique combination of 3 palladium isotopes. The barcoding allowed the simultaneous acquisition of several samples. Created in Biorender.com



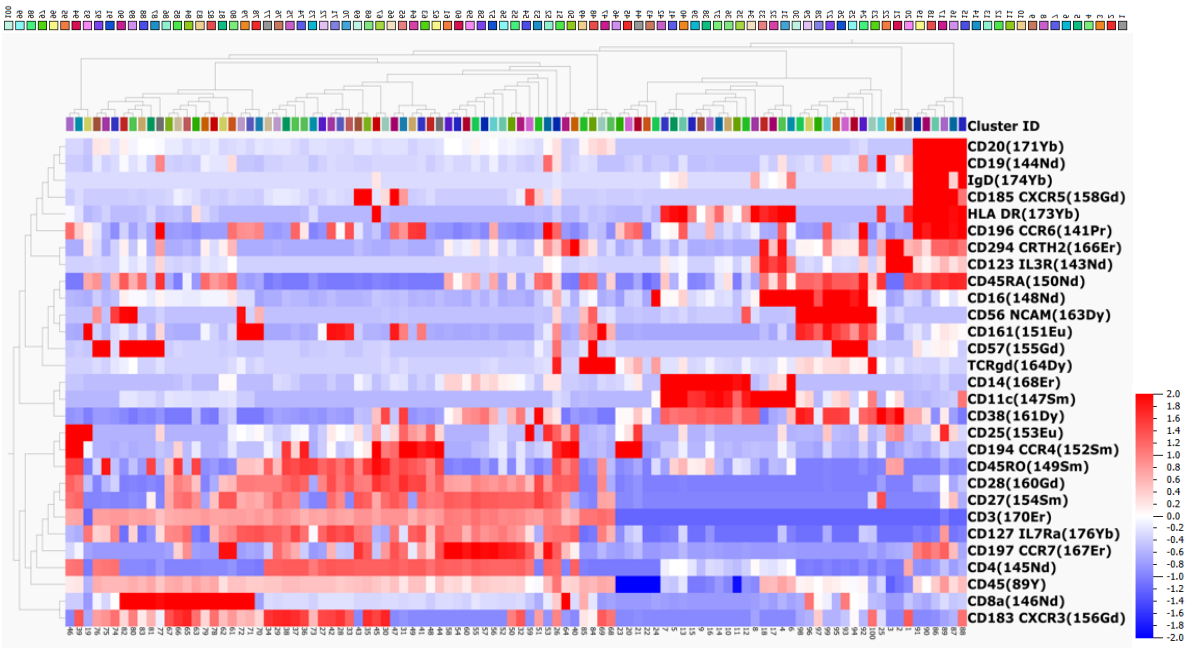
Supplementary figure 2: tSNE analysis of the reference sample pooled together to the experimental samples prior to each acquisition. After debarcoding the reference sample FCS were analysed by t-SNE and FlowSOM using all cellular surface markers. FlowSOM 100 clusters are shown by a blue to red color scale. Acquisition date is indicated at the top of each graph.



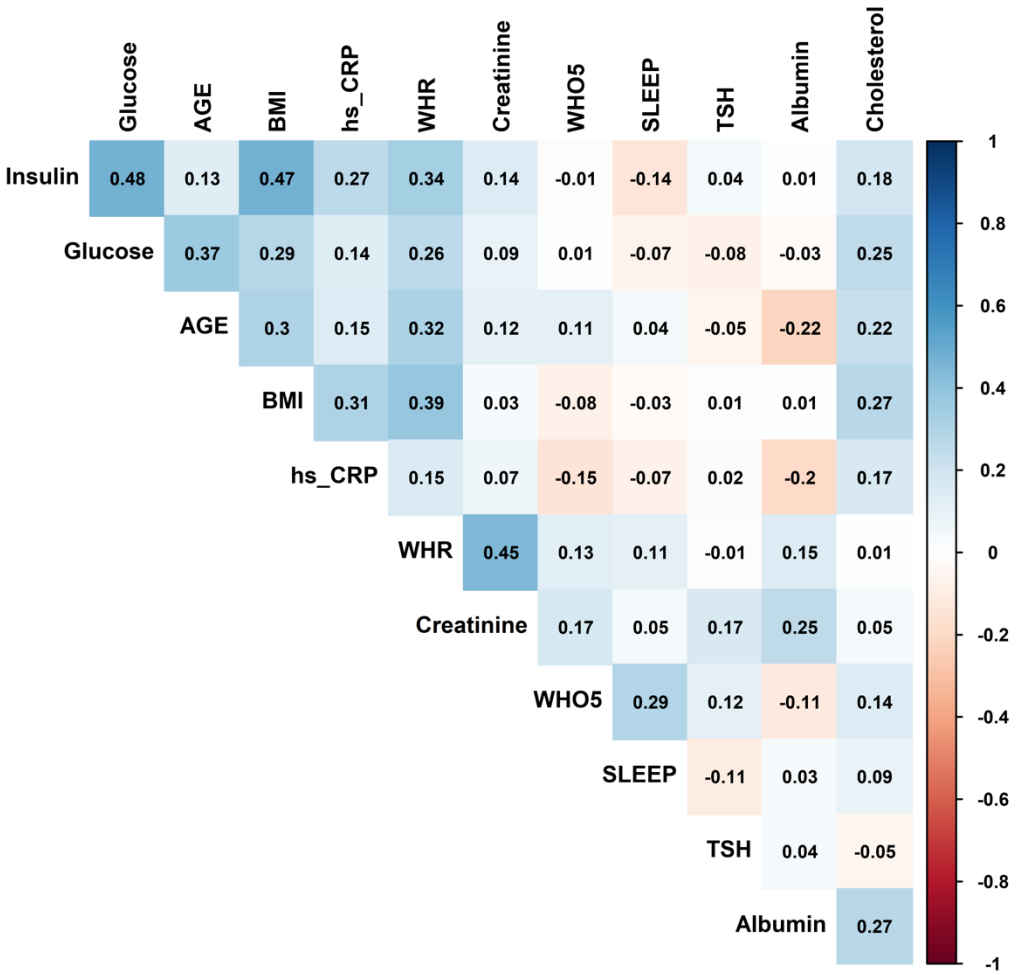
Supplementary figure 3: FlowJo Gating strategy to identify and characterize 72 relevant immune cell populations. The supervised, manual analysis of the acquired data was done based on this gating strategy, which was developed specifically for the data in question.



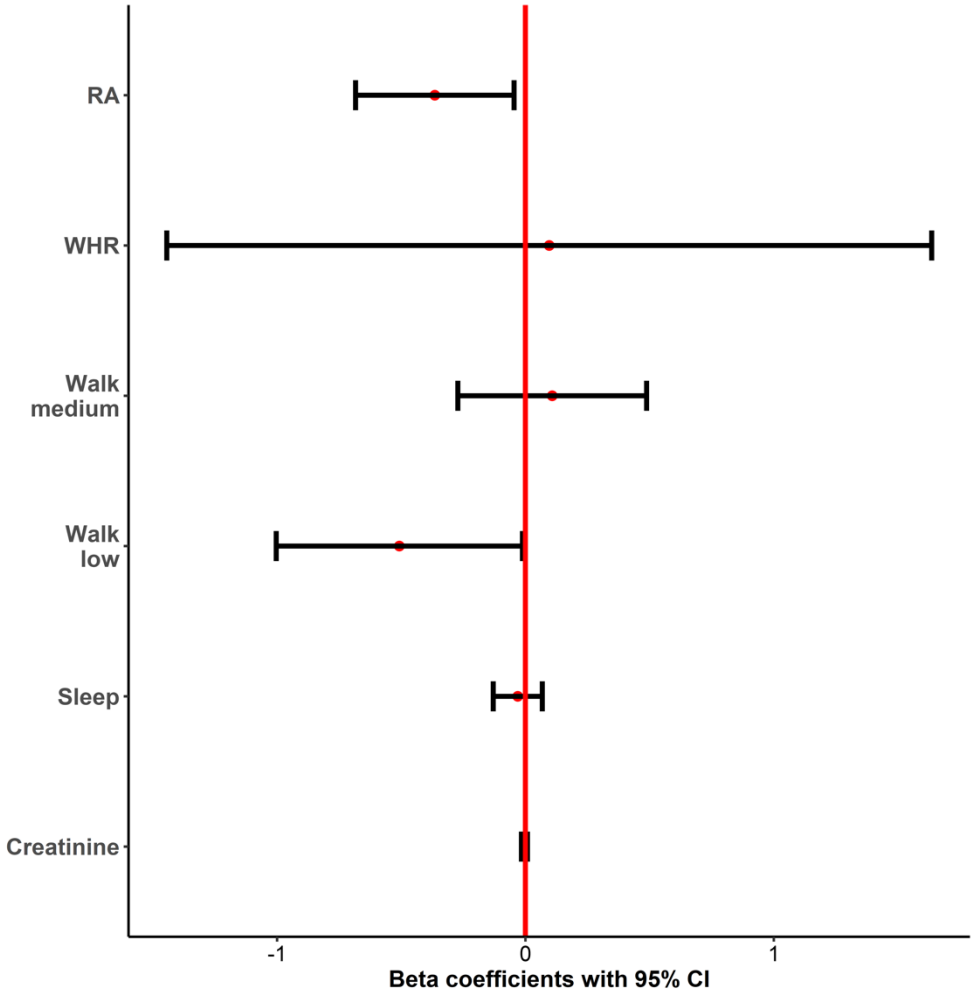
Supplementary figure 4: Flowchart of statistical analysis, showing the different steps of the statistical analysis that has been done including the final number of participants that were considered for the analysis.



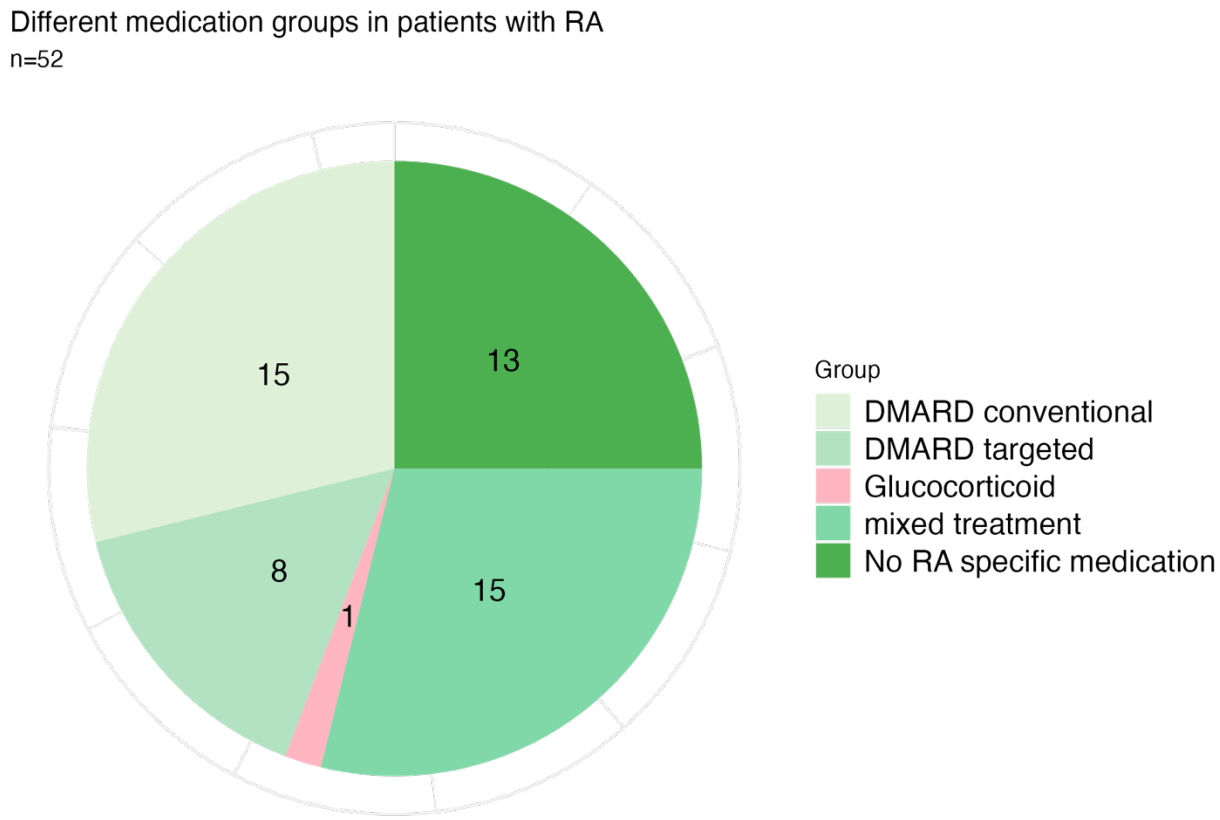
Supplementary figure 5: Unsupervised clustering analysis of differentially expressed cell markers in patients with RA and healthy controls. 10x10 cluster analysis generated by CellEngine, represented as heatmap. Color grade from red (high expression) to blue (low expression). The cell markers are listed on the right.



Supplementary figure 6: Spearman correlation for selected covariates to ensure independence of possible confounding factors. The color gradient goes from dark red (strong negative correlation) to dark blue (strong positive correlation).



Supplementary figure 7: Impact of different factors on unswitched memory B ($_{m}$B) cell frequency in patients with rheumatoid arthritis (RA) and healthy controls (HC). RA is the primary and only significant factor explaining up to 17.9% of the variance of unswitched $_{m}$B cells in this model. WHR, waist-hip-ratio; Walk, medium and walk, low; a medium and low frequency of walking for longer than 10 min; sleep, hours of sleep; Creatinine, creatinine levels µmol/L.



Supplementary figure 8: Different treatment groups. Patients with RA were grouped into five different treatment groups according: conventional DMARDs, biological and targeted synthetics DMARDs, glucocorticoids, mixed treatment and no specific RA medication. N=52. The mean days per dose per group were as follows: DMARD conventional = 6.26 days; DMARD targeted = 14.14 days and Glucocorticoid 1.56 days.

4. Discussion

The discovery of a reduced number of unswitched memory B cells in the peripheral blood of patients with RA is a significant finding in understanding the immunopathology of this chronic autoimmune disease. Unswitched memory B cells which are characterized by the expression of CD27, IgM and IgD antibodies play a critical role in the immune system by maintaining long-term immune memory and facilitating rapid responses to pathogens. A diminished population of these cells in RA patients suggests a potential impairment in the immune memory response which may contribute to the chronic inflammation and autoimmunity observed in RA. This reduction could also indicate an aberrant immune regulation, where the normal process of immune surveillance and tolerance is disrupted, possibly leading to an increased risk of infections and a diminished response to vaccines. Furthermore, these findings can inform the development of more targeted therapies aimed at restoring the balance of B cell subpopulations, potentially improving disease management and patient outcomes. The presence of altered B cell subsets in RA patients not only enhances our understanding of the disease's underlying mechanisms but also supports the development of novel personalized medicine approaches in treating autoimmune conditions. Furthermore, the reductions and increases of several cell populations, such as T regulatory cells and NK cells, before correction for multiple testing should not be neglected and need to be considered for future studies (Figure 8).

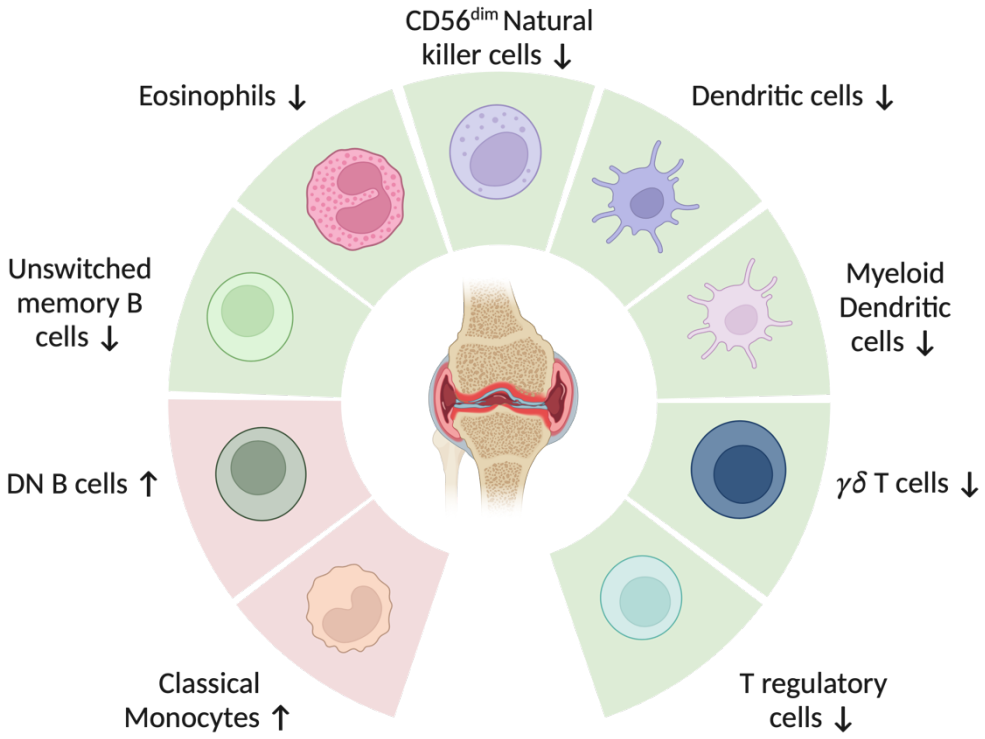


Figure 8: Differences in cell populations of patients with rheumatoid arthritis compared to healthy controls.

E. Manuscript V:

Fasting followed by time-restricted eating leads to a sustained reduction in disease activity in rheumatoid arthritis

Bérénice Hansen, Rémy Villette, Viacheslav Petrov, Cédric C Laczny, Farhad Vahid, Andreas Michalsen, Etienne Hanslian, Daniela A Koppold, Anika Rajput Khokhar, Michael Jeitler, Nico Steckhan, Brit Mollenhauer, Sebastian Schade, Torsten Bohn, Paul Wilmes, Jochen G Schneider

1. Contribution

As the first author, my contributions to this research paper included the study design, planning, execution, and analysis of all data, along with the article writing. I was supported by Farhad Vahid and Torsten Bohn for the calculation of the dietary indices calculations. For the statistical analysis I was supported by Viacheslav Petrov and Rémy Villette. The figures in the main text were generated by Rémy Villette.

2. Background and introduction

As explained in detail in the introduction, the impact lifestyle factors, such as diet and BMI on rheumatoid arthritis (RA), has garnered significant attention due to their potential influence on disease progression, treatment outcomes, and comorbidities. Obesity is recognized as a risk factor for the development of RA, with adipose tissue secreting inflammatory cytokines that may play a critical role in the disease course. Elevated BMI has been linked to increased levels of CRP, a marker of inflammation, and patients with higher BMI often experience reduced efficacy of disease-modifying antirheumatic drugs (DMARDs). Additionally, common RA comorbidities such as cardiovascular diseases (CVDs), T2D, and metabolic syndrome further deteriorate the quality of life for RA patients, exacerbating joint pain and limiting physical activity.

Weight loss interventions have generally been associated with positive outcomes in both disease activity and quality of life for RA patients. However, nutrition impacts health beyond mere weight control, necessitating a broader dietary approach. Key dietary factors such as fibre, phytochemicals, and omega-3 fatty acids might exhibit anti-inflammatory properties beneficial for RA and should be further studied. Although some long-term restrictive diets like ketogenic or low-carb diets have shown beneficial health effects and a reduction in systemic inflammation, they often fail due to poor patient adherence, limiting their effectiveness. As previously mentioned, PF has also been reported to be beneficial in RA, however, these benefits are usually reversed upon food reintroduction and the underlying mechanisms are unknown. IF has emerged as a promising dietary intervention for individuals with autoimmune diseases, including RA. IF involves alternating periods of eating and fasting, with popular protocols such as the 16:8 method or ADF. The potential benefits of IF for RA are attributed to its effects on inflammation and metabolic health. Importantly, IF is less restrictive in terms of nutrient intake, as patients are only limited by eating times rather than food choices, making this dietary pattern more sustainable and appealing for patients. Overall, IF presents a non-restrictive dietary strategy that may improve disease outcomes and quality of life in RA patients through its multifaceted impacts on inflammation and metabolic health.

The aim of this study was to analyse the impact of one week of prolonged fasting according to the Buchinger procedure, followed by twelve months of time-restricted eating according to the 16:8 method in 30 patients with RA. One objective was to maintain the beneficial impact of PF on RA that has been reported in previous studies but is usually reversed upon food reintroduction. A second objective was to observe the impact of TRE on RA disease activity, well-being of patients and their overall quality of life. A wide range of dietary information has been recorded to ensure capturing any unforeseen changes in the dietary habits of the patients. Several specific dietary indices have been

calculated, including the dietary inflammatory index (DII)[112], the MDScale[142] and the MedDiet index[143]. The indices have been adapted according to the data collected for our study.

The article introduces the scientific background of the clinical intervention trial, goes into a detailed analysis of the collected data and applies different analytical methods to represent and elaborate changes observed in the disease activity, the well-being of the patients and changes in dietary patterns.

3. Manuscript

Fasting-driven suppression of disease activity in rheumatoid arthritis

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Abstract

We investigate the combined impact of prolonged fasting and time-restricted eating (TRE) on rheumatoid arthritis (RA). Participants experienced sustained reductions in RA Clinical Disease Activity Index (CDAI), decreases in Body Mass Index (BMI) and improved adherence to a Mediterranean diet. Statistical analysis revealed that reductions in BMI, adherence to TRE and MD significantly mediated the CDAI improvements. Our findings underscore the potential of fasting-based strategies for managing and preventing RA.

Main

Fasting is defined as the voluntary abstinence from caloric ingestion for a limited time and has been linked to several health benefits in the past, which have, however, not been mechanistically understood[1]. Popular fasting methods include prolonged fasting (PF) lasting from four to 21 days with an energy intake below 350 kcal/d, and time-restricted eating (TRE), whereby food intake is restricted to a daily eating window[1]. Recent high-quality research has yielded promising results associating several underlying mechanisms to the health benefits of fasting. Some have been attributed to enhanced mitochondrial function, increased autophagy, gut microbiome changes and an overall reduced inflammation[2,3]. Thereby, fasting has been proposed to be efficacious in alleviating clinical symptoms in human chronic diseases with inflammatory signatures such as rheumatoid arthritis (RA). RA is a multifactorial chronic and systemic auto-immune disease, affecting 1% of the global population with women being at a higher risk than men[4,5]. The disease typically impacts the synovial lining of the joints but also entails various comorbidities, significantly reducing their quality of life[6]. Fasting has been previously reported to be beneficial in RA and within the framework of the ExpoBiome study, a clinical cross-sectional and longitudinal intervention trial, we aimed at investigating the effects and underlying mechanisms of one week of PF followed by 12 months of TRE in patients diagnosed with RA[7].

Results

In our present study, we included 30 patients with RA (90% female) in the longitudinal study. Baseline characteristics are provided in Supplementary Table 1.

We aimed at assessing changes in clinical disease activity index (CDAI) and clinico-anthropometric parameters over time compared to the baseline. In this context, a statistically significant decrease was observed for the CDAI, with the lowest being recorded immediately after the PF, but with a significant decrease remaining for the whole intervention ($p < 0.05$, Figure 1A). Importantly, we observed a statistically significant shift from the high activity to the low activity category, especially at early stages of the intervention ($p < 0.0001$, Figure 1B). This finding was accompanied by several improvements in relation to questionnaires concerning well-being ($q < 0.05$, Supplementary figure 1). In addition, we noted several sustained anthropometric changes over the course of the intervention. More specifically, a significant decrease was recorded for BMI and hip circumference until week 52 ($p < 0.01$, Figure 1C), while a reduction in diastolic and systolic blood pressure (BP), as well as waist circumference was sustained until week 26 ($p < 0.01$, Figure 1C). An increased heart rate was observed during the PF period ($p < 0.01$, Day 6, Figure 1C). In addition, we noted a decrease in glucose, HbA1C, total cholesterol, insulin and CRP ($p < 0.05$, Figure 1C), and a reduced blood count for erythrocytes, leukocytes, lymphocytes, neutrophils and reticulocytes ($p < 0.05$, Figure 1C). An increased basophil count was noted at week 52 ($p < 0.01$, Figure 1C). Moreover, we observed an increase for alanine transaminase (ALT) at Day 6 and Day 8-12, which was then followed by a decrease in week 26 ($p < 0.05$, Figure 1C).

The TRE intervention was accompanied by spontaneous and unsupervised dietary shifts towards an increase in ovo-lacto-vegetarian- and vegan-like food intake during the study ($p < 0.0001$, Figure 2A). Moreover, adherence to overnight fasting was significantly increased at all stage of the intervention ($p < 0.001$, Figure 2B). Hence, we calculated two different mediterranean diet indexes, namely the MDScale and MedDiet scores, based on the questionnaire data. Both scores indicated a shift from a

Western diet towards an adherence to a MD diet over the course of the intervention ($p < 0.01$ Figure 2C). Taking into consideration these dietary shifts, we applied a linear mixed-effects model analysis, showing that BMI, MD adherence and overnight fasting had a significant effect on CDAI (Figure 2D).

Discussion

Our data show profound impacts of fasting and diet on RA over time, highlighting the combined effect of weight loss, dietary adaptations and fasting. Contrary to previous findings [8], the decrease in CDAI induced via PF, sustained by TRE, lasted for as long as 12 months in our present study. This decrease was accompanied by distinct biochemical changes reflecting reduced inflammation. While general dietary guidelines for RA primarily focus on specific nutrients and food items, such guidelines underappreciate overall dietary composition and meal timing[9]. Our findings highlight the significance of meal timing in relation to the body's circadian rhythm through strategies such as TRE.

The observed beneficial effects may be the results of various mechanisms at play, including enhanced ketogenesis, improved mitochondrial function, increased autophagy, modulated gene expression, weight reduction, reduced apoptosis, and decreased inflammation[1,10,11]. These are reflected in the improved metabolic profile of the patients observed after fasting, including improved blood glucose and cholesterol levels as well as a reduced CRP. Fasting induced gut microbiome modulation might also be a key mediator of the impact of TRE and PF on RA[12]. Nonetheless, nutritional composition remains vital for RA disease management. The observed higher adherence to a MD was previously reported as potentially beneficial in patients with RA, leading to a reduction in pain and improved physical function, partially mediated by an increased fiber intake and the resulting beneficial effect on the gut microbiome[13,14]. However, evidence to generally recommend a MD for patients with RA has been assessed insufficient in 2018 [14-19]. In this context, the present study is highly relevant, and it underscores the importance of integrating both quality and timing of diet into RA management. By combining and implementing various dietary strategies, namely PF followed by a maintenance diet consisting of TRE and a higher MD adherence, patients with RA could optimize health outcomes and benefit from personalized nutrition interventions tailored to their individual needs.

Study limitations

The unexpected dietary adaptations in relation to MD might impact the observed benefits of PF and TRE. Investigating whether combining TRE with a MD or a specific anti-inflammatory diet yields greater improvements require further investigation.

Methods

Detailed method descriptions and explanations are provided in Supplementary Information. The study intervention consisted in 30 patients with RA undergoing one week of PF which was followed by 12 months of TRE. Patients attended a total of 11 visits, and at each timepoint sample and data collection took place. The samples for routine blood chemistry were collected at the Charité-Universitätsmedizin Berlin and measurements were performed after each visit. Blood samples were taken at a baseline visit (Day 1), on day six of the PF and on nine additional visits during the TRE over 12 months[7]. Several questionnaires assessing clinical and nutritional data were answered either on site during the visit or prior to the visit at home and captured in REDCap (Supplementary Table 2). Most patients (70%) were undergoing a typical RA treatment over the total course of the study, consisting of conventional synthetic disease-modifying antirheumatic drugs (csDMARDs, 50%), or biological DMARDs (bDMARDs, 27%), either alone or in combination with csDMARDs or glucocorticoids. No significant changes in the treatment regimens were observed during the 12 months of the study.

Two different scores for the adherence to a MD diet were calculated to assure an appropriate usage and coherence. The choice of the two MD indices, namely the MDScale and the MedDiet score[20,21], was based on an extensive literature review comparing five MD indices published in 2019 by Aoun et al.[22]. The calculation of the indices was slightly adapted (Supplementary information). For the MDScale, nine items were included and calculated based on a gender-specific median amount, while for the MedDiet score, the score was calculated based on their frequency of consumption per week. Several food items led to a higher score, including vegetables, legumes, fruits, nuts, cereals and fish, while dairy products, meat and alcohol were classified as negative items.

The clinical and nutritional data collected in REDCap alongside the molecular data were analysed and integrated using R (R 4.3.2, RRID:SCR_001905) and R studio (2023.09.1+494, RRID:SCR_000432). The CDAI is calculated based on the swelling and pain of the joints of the patients and divided into different severity categories, defined as: Remission [0,2.8], Low [2.8,10], Medium [10,22] and High [>22]. A

repeated measures ANOVA test or Friedman test (depending on the normality of the distribution) was performed to identify any significantly divergent values over the different timepoints and corrected with FDR. For the parameters for which the repeated measures ANOVA/Friedman tests showed a significant value, Student/Wilcoxon tests for paired values were applied. The post hoc test p values were not corrected as correction was already applied on the repeated measures tests. A Cochran test was applied for the CDAI. Wilcoxon, Friedman, Student and Cochran tests were performed using the *rstatix* package (v0.7.2, RRID:SCR_021240). Chi-square tests were performed using the stats R package (v4.4.2, RRID:SCR_025968). Mixed linear modelling was done using the package lme4 (v1.1.35.5, RRID:SCR_015654) and lmerTest (v3.1.3, RRID:SCR_015656) in R was applied[23,24].

Figures

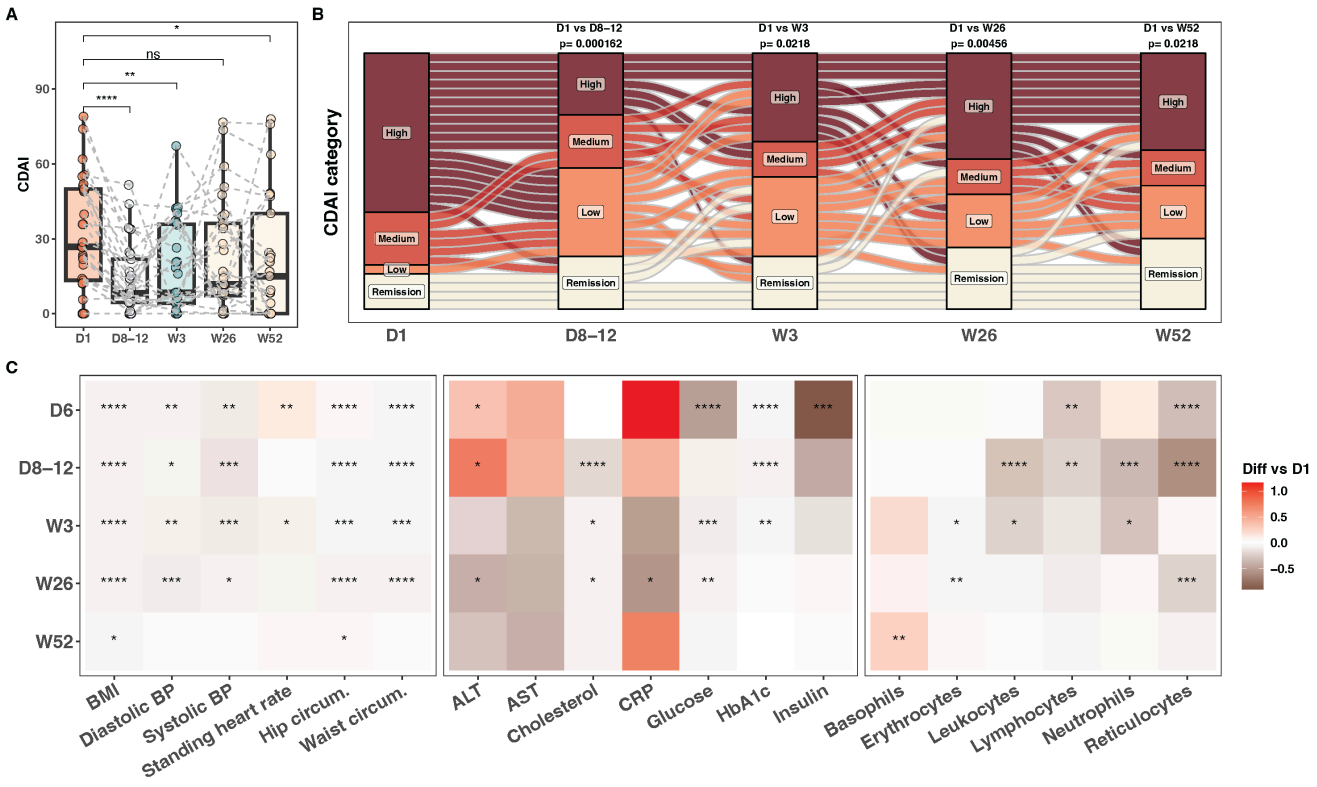

Figure 1: The impact of prolonged fasting (PF) followed by time-restricted eating (TRE) on several health outcome in patients with RA.

A. Changes of clinical disease activity index (CDAI) over 12 months. P-values represent Wilcoxon test results. **B.** Alluvial plot showing the CDAI category evolution after PF and over 12 months of TRE. P-values are based on a Chi-square test comparing CDAI values from the individual timepoints to those of the baseline (Day 1).

C. Heatmaps representing the differences of mean values compared to the baseline. Heatmaps are showing differences in anthropomorphic evolution (left), blood biochemistry parameters (middle) as well as blood cell counts (right).

*p<0.05, **p<0.01, ***p<0.001, **** p < 0.0001

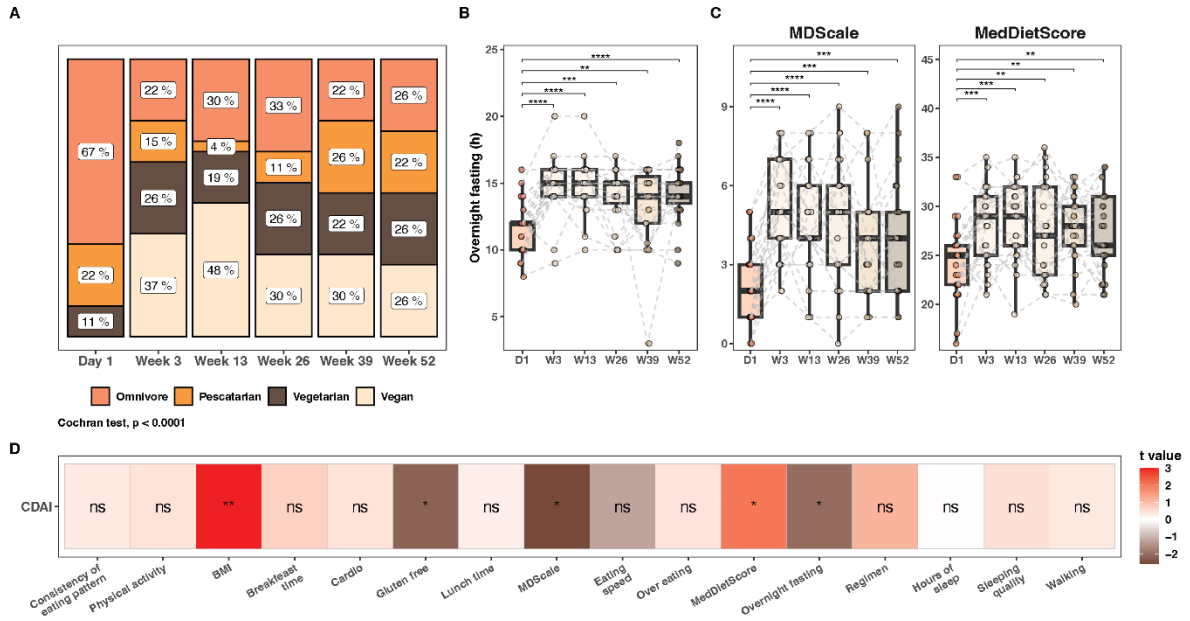

Figure 2: Prolonged fasting (PF) and time-restricted eating (TRE) were linked to dietary changes during the 12 months of intervention.

A. Change of dietary patterns changes over the intervention period. A Cochran test was conducted. **B.** Change of overnight fasting in hours over the time of the intervention. **C.** Boxplots for the MDScale and MedDiet score over the intervention period. P-values are Wilcoxon test results corrected with FDR. **D.** Figure representing the magnitude of impact of dietary and lifestyle factors on clinical disease activity index (CDA), including the consistency of eating patterns, the level of physical activity, body-mass index (BMI), dietary composition such as a gluten-free diet or an adherence to a Mediterranean diet (MDScale, MedDiet score) and the overall composition of the regimen (omnivore, pescetarian, vegetarian, vegan), as well as the duration of the overnight fast and the hours of sleep and response variable CDAI. *p<0.05, **p<0.01, ***p<0.001,

Data availability

The clinical data used in this study is held by the clinical partner and is not shared publicly due to privacy and confidentiality agreements. Access to this clinical data may be granted upon direct request to the corresponding author, subject to their approval and ethical considerations.

Code availability

All code used for the data analysis can be found in the following repository: gitlab.lcsb.uni.lu/TBD.

Trial registration number and date at clinicaltrials.gov:

NCT04847011, April 14, 2021

Ethical approval and consent

Ethical approval for this study was from the institutional review board of the Charité-Universitätsmedizin Berlin (EA1/204/19) and the Ethics Review Panel (ERP) of the University of Luxembourg (ERP 21-001-A ExpoBiome). Participants were included into the study only after having voluntarily provided written informed consent [7].

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Author contributions

Study design and protocol: B.H, C.C.L, J.G. S, P.W; the conceptualisation of the intervention: E.H, D.A.K, A.M, A.R.K, B.M, S.S, N.S, J.G.S, P.W; clinical trial and sample collection design and administration by B.H, E.H, D.A.K, A.M, A.R.K, B.M, S.S; funding acquisition: C.C.L, P.W.; statistical analysis, calculation of

the DII, MD indices was done by B.H, F.V, V.P, R.V; data visualisation: R.V; sample size calculation: C.C.L, J.G. S, P.W, K.R; initial draft writing: B.H, R.V; sample protocol preparation: B.H; manuscript review and editing: J.O.S, P.W; all authors contributed to, read and approved the final manuscript.

Competing interests

Authors declared no competing interests.

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Fasting-driven suppression of disease activity in rheumatoid arthritis – Supplementary files

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TABLES

Supplementary table 1: Baseline characteristics of the study cohort

n=30	Median	IQR
Age(y), mean, SD	55.88	10.16
Female [%]	90.00	NA
Disease duration	4.75	8.56
Weight [kg]	69.50	20.6
BMI [kg/m2]	25.02	7.22
WHR	0.81	0.07
Systolic blood pressure	127.57	16.67
CDAI	31.81	22.25
HAQ	0.50	0.875
Vegetarian [%]	11	NA
CRP	1.52	3.03
Medical Treatment [%] *	70	NA

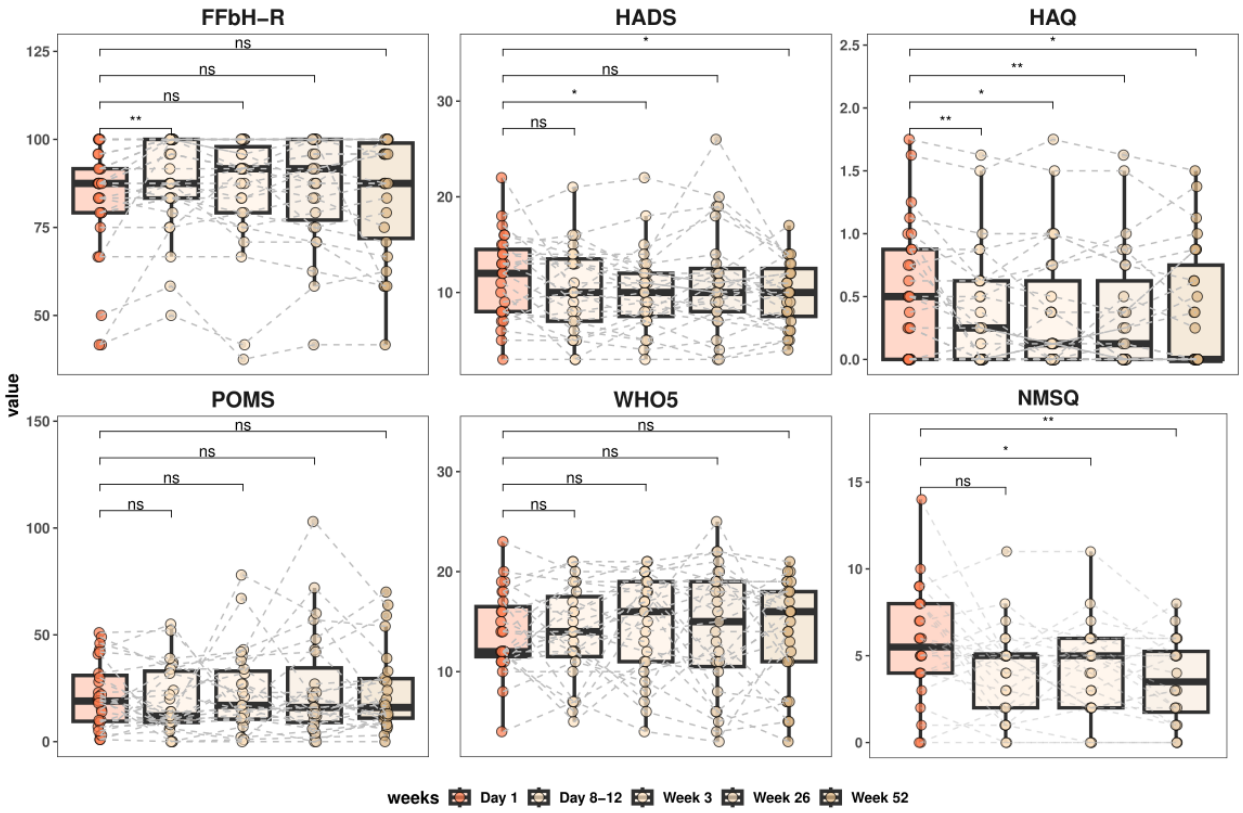
Abbreviations: BMI, body mass index; WHR, waist-hip ratio; CDAI, clinical disease activity index; HAQ, health assessment questionnaire; CRP, C-reactive protein; IQR, interquartile range.

*Either DMARD, NSAID, corticosteroid or combined treatment

Supplementary table 2: Health assessment questionnaires

Disease specific
• Hannover Functional Ability Questionnaire (FFbH-R) • Clinical Disease Activity Index (CDAI)
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) • Bristol Stool Scale • Quality of Life questionnaire (WHO-5) • Hospital Anxiety and Depression Scale (HADS) • Profile of Mood States (POMS) • Non-Motor Symptoms Questionnaire (NMSQ)

FIGURES



Supplementary figure 1. Additional scores measured during the intervention.

Changes reflected in the answers to the different health questionnaires over time. From left to right: Hannover Functional Ability Questionnaire (FFbH-R), Hospital Anxiety and Depression Scale (HADS), Profile moods of states (POMS), Quality-of-Life Questionnaire (WHO-5) and non-motor symptoms questionnaire (NMSQ). Each dot represents an individual patient, with the dots connected by a dashed line indicating the longitudinal changes observed for the same patient. *P*-values are derived from a paired Wilcoxon test for all timepoints against the baseline (Day 1).

Supplementary information

Calculation of the dietary inflammatory index (DII)

The DII was calculated based on the following 23 food components or nutrients: Alcohol, coffee, carbohydrate, cholesterol, energy, total fat, folic acid, garlic, ginger, iron, magnesium, multi-unsaturated fatty acids (MUFA), saturated fat, niacin, protein, riboflavin, selenium, thiamine, vitamin A, C, D, E and green or black tea. The data was taken from both 24FR and FFQ[1], which were adapted for the use in this study .

Calculation of MD scores

The first score that was calculated for MD adherence was the MDScale, which is the oldest MD adherence assessment index and was developed in 1995 and revised in 2003, using sex-specific median calculations and a total of 9 food groups[2,3]. The second score used in this study was the MedDiet score, developed in Greece in 2005 as an alternative to the gender-specific MDScale and has been widely used ever since[2,4]. For the MDScale, nine items were included and calculated based on a gender-specific median amount, namely vegetables, legumes, fruits, nuts, cereals and fish as beneficial items and dairy products, meat including poultry and alcohol as negative items. This resulted in a score between zero and nine, with nine indicating the highest adherence to an MD diet. Contrary to the original MDScale, the mono-unsaturated fatty acid to saturated fatty acid ratio was not considered as this data was not collected during the study. For the MedDiet score a total of eight items was included and the score was calculated based on their frequency of consumption per week. Items leading to a higher adherence were cereals, fruits, vegetables, legumes, and fish, while items negatively impacting the MedDiet score were dairy products, meat and alcohol. In this index, potatoes, olive oil and poultry were not included. The adapted MedDiet score calculated in this study ranges from 0 to 40, with 40 indicating a high MD adherence.

Statistical analysis

A first mixed-effect model was applied for the primary outcome parameters captured in the health questionnaires. Mixed effect models contain both fixed effects and random effects and are particularly useful when dealing with data that have multiple levels of variability such as our data[5]. The fixed effects are constant across individuals and the primary variables of interest in the study

while the random effects account for variation across different levels of the data and are not of primary interest[5]. The model used to analyse the effects of various effects on the different health outcomes was as follows: Health outcome ~ MDScale + MedDietScore + BMI + regimen + overnight_fast_duration + sleep_hour + gluten_free + `consistency of eating pattern` + `Physical activity` + (1 | Record_id). A second model was built with BMI as response, e.g. BMI ~ overnight_fast + meat + (1 | Record_id). The model as was also applied to the MD indices. The p-values were corrected for multiple testing with FDR correction. Plotting was performed using *ggalluvial* (RRID:SCR_021253)*, ggrepel* (RRID:SCR_017393)*, patchwork* (RRID:SCR_000072)*, ImmuMicrobiome and gglopt2* (RRID:SCR_014601) [6,7].

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4. Discussion

The finding that RA symptoms can be sustainably reduced through dietary interventions such as fasting followed by TRE, accompanied by dietary changes, holds significant clinical relevance and underscores the importance of considering the timing of nutrition in managing RA. However, quality of nutrition cannot be dismissed as we observe significant changes in food intake, namely a reduction in animal product intake and an increase in legume consumption. Although the statistical correlation between these dietary practices and symptom relief might not always be significant, the observed pattern of improvement in patients suggests a meaningful impact. The emphasis on anti-inflammatory foods could help reduce disease activity, while the structured eating patterns in TRE may optimize metabolic and immune responses, while both approaches might beneficially impact the gut microbiome composition and function. This dual approach offers a holistic strategy for RA patients, integrating nutritional considerations into their treatment plans. This emerging evidence highlights the potential of dietary modifications as a complementary approach to traditional RA management.

The clinical relevance of these findings lies not only in the potential for symptom relief but also in offering patients with RA additional strategies to manage their condition autonomously. As RA is a chronic disease with significant variability in patient response to treatment, personalized dietary interventions like fasting, TRE, and adherence to an anti-inflammatory diet such as MD provide promising avenues for improving the quality of life. A higher adherence to a MD might support the beneficial effects of fasting and TRE, potentially creating a synergistic effect that further alleviates RA symptoms; however, this needs to be further tested. More research is needed to refine these approaches and understand the underlying mechanisms, but the current evidence supports their integration into comprehensive RA care. This approach acknowledges the role of lifestyle factors in disease management and encourages a more nuanced view of patient care.

VI. Conclusion and perspectives

Several major aspects of nutrition and fasting on clinical phenotypes in rheumatoid arthritis have been elucidated in this cumulative thesis by elaborating a large clinical trial and the analysis of its complex experimental samples. This PhD project has thus provided novel and valuable insights into the connection between fasting, nutrition and chronic autoimmune diseases such as RA.

The first major finding of this thesis project was based on the cross-sectional immunophenotyping of patients with RA compared to HC (Manuscript III). We observed a significantly lower frequency of unswitched $_{m}$B cells in patients with RA compared to healthy controls, independent of medical treatment and as only abnormality of the immunophenotype of the patients with RA. It has been hypothesized that the decrease in unswitched $_{m}$B cells in the peripheral blood might be partly due to a recruitment of these cells to the synovial membranes of patients with RA[25]. However, one must consider that both unswitched and switched $_{m}$B cells accumulate in the sites of inflammation, while a decrease in peripheral blood is only seen for the unswitched $_{m}$B cells[25]. Interestingly, early exposure to the bacterium *P. falciparum* infection resulted in a dysregulated development of B cells in children and might support a strong connection between the gut microbiome and the onset and development of auto-immune diseases mediated by a dysregulated B cell profile[144]. Some patients with RA show normalization of peripheral unswitched $_{m}$B cells after anti-TNFα therapy, suggesting that TNFα neutralization might prevent these cells from migrating into the synovium[29].

Previous findings on B cell metabolism propose that differentiation into plasmablasts need large amounts of nutrients and energy provided via the anabolic glycolysis system. The reduction in nutrient availability during fasting triggers the activation of AMPK, a central energy sensor, which leads to the suppression of the mTORC1 pathway. This suppression is critical in limiting the differentiation of unswitched memory B cells into plasmablasts, thereby reducing antibody production and dampening the autoimmune responses that drive RA pathogenesis. Therefore, one may speculate that based on the requirement of nutrient availability for differentiation of unswitched B cells via mTORC1 activation, fasting might lead to a reduction in RA activity by decreasing auto-antibody production and re-establishing a balanced B cell distribution[28]. This hypothesis could be further tested by analysing changes in unswitched $_{m}$B cell levels over the PF and TRE period of the ExpoBiome trial. Also, the mTORC1 complex regulates cell autophagy, whose upregulation might induce a hyperactivation of T cells, apoptosis resistance and an increased antigen presentation[41]. To analyse such changes in T cell activity, samples of the synovial fluid of patients with RA should be collected and analysed longitudinally.

The second essential finding of this project was the effect of PF followed by TRE on RA. It is important to note that no patients dropped out due to adverse events or lack of compliance and both PF and TRE are simple, cost-effective, and practical interventions for RA patients, with potential for long-term adherence without notable adverse effects. This is particularly significant, as many dietary or lifestyle interventions fail primarily due to poor compliance or difficulties in implementation. PF followed by TRE induced several significant beneficial changes, which have been listed in chapter V, amongst others a sustained improvement of RA symptoms (CDAI) and a decreased BMI throughout the study period of twelve months.

The implication of an increased BMI and obesity on RA have been elaborated in chapter IV, showing that a reduction in BMI and adipose tissue might be significant in the context of RA and other NCDs. While traditional CR has shown mixed outcomes, with benefits often counterbalanced by adverse effects such as cold sensitivity, menstrual irregularities, hormonal changes, and decreased bone mineral density, TRE appears to bypass these negative outcomes[145,146]. Notably, these fasting regimens not only avoid detrimental effects but have also been linked to improvements in bone mineral density, an important consideration for RA patients who are at an increased risk for osteoporosis and fractures[147]. The sustained weight loss and decreased RA activity achieved by TRE in this clinical trial might therefore also be relevant for other NCDs. In addition, the circadian factor of TRE compared to other fasting regimens should be considered. The gut microbiome has been suggested to play a major role in RA and its composition and function are underlying diurnal shifts. Hence the harmonization of dietary pattern with the circadian rhythm of the gut microbiome induced by TRE might be a key player in regulating host inflammation. A study published in Cell in 2024 confirms this hypothesis and our findings by stating that the periodicity of arthritis inflammation is mediated via the impact of dietary pattern on the gut microbiome[54]. They reported that *Parabacteroides distasonis* oscillates according to the dietary pattern of the host and mediated SIRT5 inflammation via the production of glycitein[54].

The change of food choices should, however, not be neglected. Although the focus of this clinical trial was merely on food timing, patients adapted their diet, leaning towards a higher MD adherence. This might be based on several factors, including a possible influence of the environment where patients have been recruited and an increased health awareness due to the participation in a clinical trial. Also, the circadian alignment and possible gut microbiome alterations might subconsciously change dietary choices to some extent, as consistent eating schedules have been associated with reduced cravings. Another factor might be that some patients skipped a meal to comply with the reduced eating window and consumed a higher amount of food at home as compared to before the study and prior to TRE. This altered eating pattern might therefore result in a higher consumption of fresh, plant-based foods

compared to processed take-away meals on the go. However, although the MD adherence increased, the diets consumed by the patients with RA cannot be necessarily classified as MD.

The possible effects of these changed dietary pattern on the gut microbiome, including both timing and food quality, might be of major significance. Consistent eating schedules, reduced eating windows and diets high in fibre and phytochemicals have been previously reported to act beneficial on the gut microbiome composition and function, leading to a higher SCFA production and increased gut barrier function amongst others. Also, the oral microbiome is closely linked to the gut microbiome and *P. gingivalis* can induce citrullination.

The according samples collected during the ExpoBiome study will hopefully lead to valuable insights about the impact of PF, TRE and decreased intake of animal products on the gut microbiome.

In summary, PF and TRE, through their multifaceted effects on metabolism, immune regulation, inflammation and circadian rhythm, offer promising strategies for modulating the immune system in RA. Fasting, combined with other lifestyle changes, could potentially lead to improved disease outcomes without the adverse effects associated with traditional medical therapies (Figure 9).

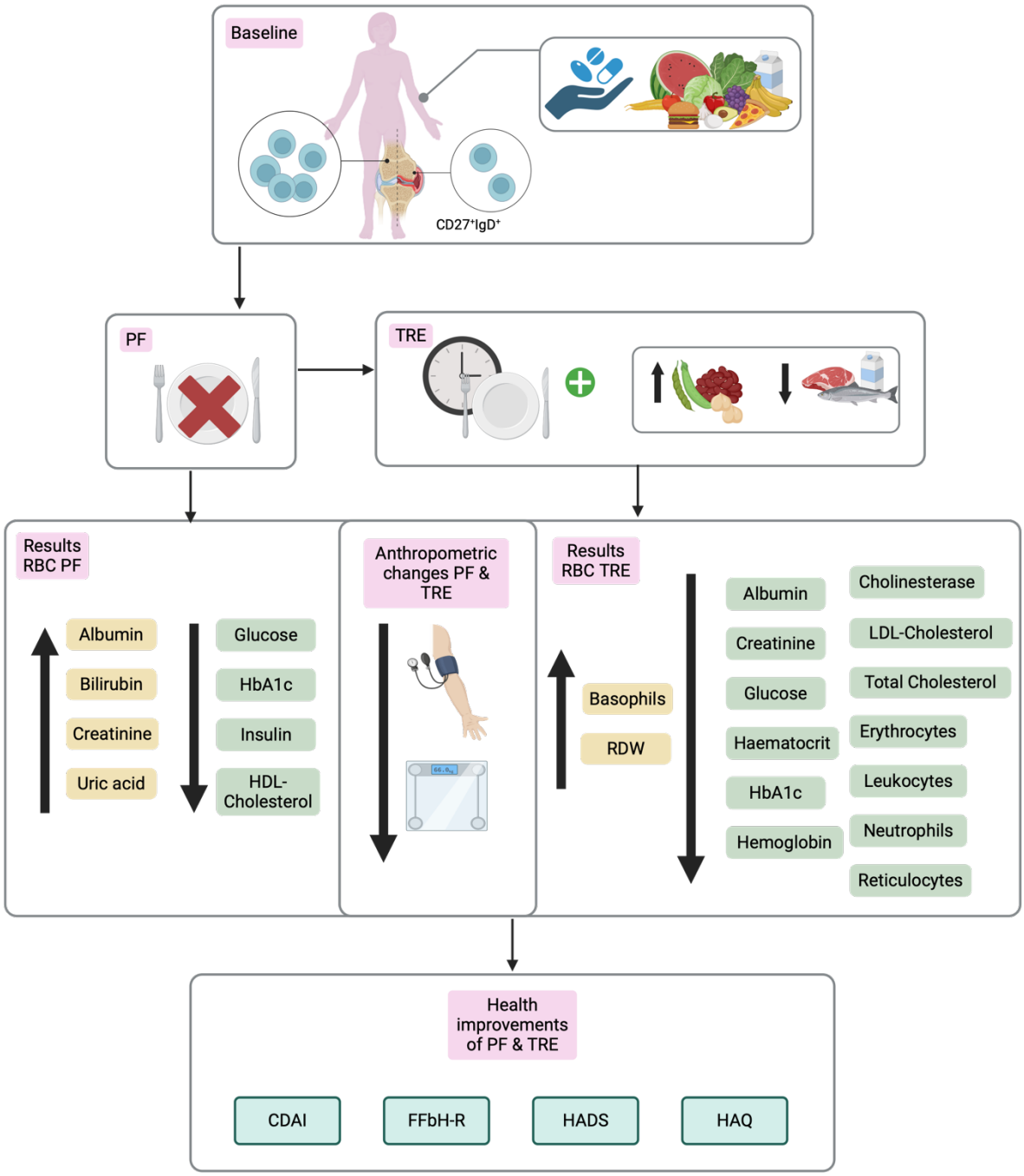

Figure 9: Summary of results.

Baseline: Patients with rheumatoid arthritis (RA) have a lower frequency of peripheral CD27+ IgD+ memory B cells compared to healthy controls at baseline. At baseline most patients were consuming an omnivore diet and not treatment naïve. PF: The second step of the study was one week of prolonged fasting (PF), where 30 patients with RA followed PF according to the Buchinger procedure for 7 days. Results RBC (Routine blood chemistry) PF: PF led to several anthropometric and biochemical changes. TRE (Time-restricted eating): In the next phase of the study, patients with RA followed a TRE pattern, according to the 16:8 method for 12 months. During these 12 months, the patients also decreased their intake of animal products while their consumption of legumes was increased. Results RBC TRE: This intervention also lead to several changes.Anthropometric

changes PF & TRE: Both PF and TRE led to a decrease in weight and blood pressure. Health improveme,nts of PF & TRE: The combination of several different effects of PF and TRE led to an improvement in clinical disease activity and wellbeing in the patients with RA, captures in the clinical disease activity index (CDAI), the health assessment questionnaire (HAQ), the Hospital Anxiety and Depression scale (HADS) and the Hannover Functional Ability Questionnaire (FFbH-R).

Overall, this project shows a very solid foundation on RA immunology and the effects of different dietary interventions, namely PF and TRE on the pathophysiology. However, although major findings refining the picture of RA have resulted out of this PhD project, including the decreased IgD+CD27+ $_{m}$B cell frequency in patients with RA, beneficial effects of PF and TRE on CDAI, sustained weight loss over 12 months and the beneficial effect of a MD on RA, more research is needed. Despite a very low dropout rate of the study participants, the number of 30 patients is still low considering that both males and females were included. Also, due to the wide age span, we might not have been able to control for age specific effects. The patients did serve as their own control, which emphasizes the reliability of our results, but focusing on a specific age group might be more beneficial for personalised nutrition approaches. Also, as most patients with RA in our study, and globally, are women, hormonal changes and fluctuations should be taken into consideration. We did not record menstrual cycles or reproductive hormonal fluctuations of the participants. As symptoms as pain and mood do often significantly change over the course of a menstrual cycle and oestrogens have been suggested to be implicated in the RA disease pathogenesis, this might impact the reported results.

Based on our findings and the limitations of the ExpoBiome study, I would suggest a follow-up randomized controlled trial. In addition to the inclusion and exclusion criteria applied during ExpoBIome study, excluding male participants and focusing on female patients with RA with similar age and hormonal status, might be very insightful. Focusing on patients with RA who are either currently employed or retired, rather than both subpopulations should be considered. This dissection of subpopulations might lead to a more homogenous timing of the eating windows during TRE. In general, currently employed and therefore often younger patients tend to show a preference towards a later eating window while the older retired patients generally prefer an earlier eating window. One issue we encountered during the ExpoBiome trial was the unintended shift to a higher adherence of a MD in patients following the TRE pattern. A possibility to avoid a mix of different interventions could be to eventually offer both to the study participants. By giving them clear guidance for either first TRE followed by MD or vice-versa patients might be more inclined to strictly follow the guidelines of the clinical study. Therefore, the follow-up trial should consist of at least three different study arms. After undergoing one week of PF, like the ExpoBiome trial, one arm would continue the dietary intervention by applying six months of TRE, while a second arm would implement a MD. After six months both arms would receive further dietary guidance, instructing them on either TRE or MD to now implement both

TRE and MD for another six months. A third arm should figure as a control group and not undergo any dietary intervention. Possible adaptations to a healthier lifestyle in the control group might be anticipated by offering them the same dietary guidance after the end of the study. To avoid any bias, patients should be randomly attributed to each of these arms and their dietary habits should be assessed in a lead up week after assessment of baseline parameters by lifestyle and dietary questionnaires. The food recording could be optimized by using AI tools quantifying the dietary intake of the patients rather than classical 24HFR reporting In addition to the clinical, nutritional and anthropometric data collected in the ExpoBiome trial, a record of the menstrual cycle should be included as well as the DAS28 questionnaire in addition to the CDAI. Such a design would offer the possibility to discern the effects of a MD diet from a TRE alone and avoid selection bias by including the control group. In addition, it would offer a chance to analyse the role of female reproductive hormones in connection with nutrition and clinical disease activity in RA. Although clinical trials of a similar scale are highly expensive and require a strong commitment from both the researchers and the study participants, results would be of high importance and help elucidate the underlying mechanisms of the impact of nutrition on RA.

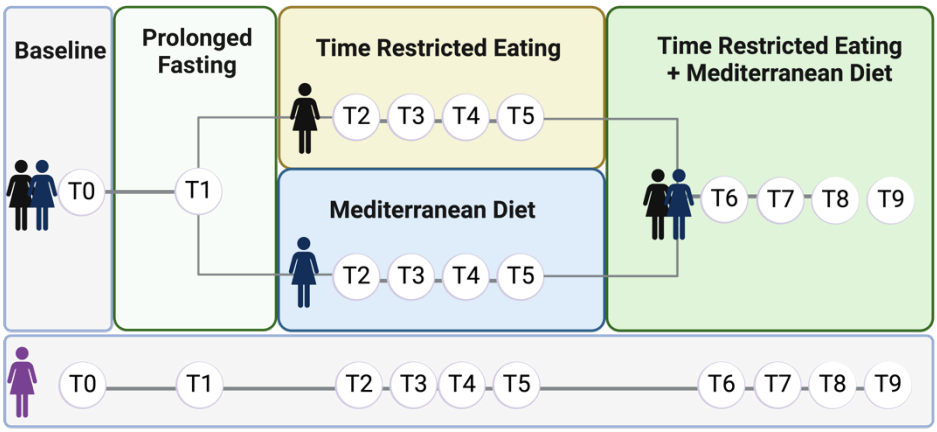

Figure 10: Proposed study design for follow-up trial.

This study design shows a clinical intervention trial analysing the effects of prolonged fasting (PF), time-restricted eating (TRE) and a mediterranean diet (MD) on the chronic and systemic auto-immune disease rheumatoid arthritis (RA). The study design includes three different study arms, two undergoing PF followed by eiterh TRE or MD for six months and then combining both interventions for another six months, while a third arm solely figures as controls and does not undergo any intervention. The different visits would be as follows: T0 baseline, T1 during the 7 day prolonged fast, T2-T4 spread across 6 months of TRE vs. MD intervention and an additional four study visits T6-T9 equally distributed across the six months of the combined dietary intervention.

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