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Intermittent Fasting Cohort Study

"InterFast"

Ethics number: 27-166 ex 14/15

Protocol code: HS – 2014 – 03

Date and version number: 01/06/2018 Version 1.9

Registration with ClinicalTrials.gov: NCT02673515

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CONFIDENTIAL

This document contains confidential information that must not be disclosed to anyone other than the sponsor, the investigator team, regulatory authorities and members of the Research Ethics Committee.

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2. LIST OF ABBREVIATIONS

AIx *Aortic augmentation index*

BMCyt *Buccal Micronucleus Cytome*

DBP *diastolic blood pressure*

DEGS *German Health Examination Survey for Adults*

DEXA *dual-energy x-ray absorptiometry*

ECG *Echocardiogram*

EDTA *ethylenediaminetetraacetic*

FFQ *Food frequency questionnaire*

FMD *Flow mediated dilatation*

FPFV *First patient first visit*

IC *indirect calorimetry*

IMT *Intima media thickness*

IPAQ *International physical activity questionnaire*

LPFV *Last patient first visit*

LPLV *Last patient last visit*

MN *micronucleus*

NaCl *Sodium Chloride*

NEMONIT *German National Nutrition Monitoring*

OGTT *oral glucose tolerance test*

PAT *peripheral arterial tone*

PWA *Pulse wave analysis*

PWV *pulse wave velocity*

RCT *Randomised controlled trial*

REE *resting energy expenditure*

RHI *reactive hyperemia index*

SBP *systolic blood pressure*

Tris HCl *Tris hydrochloride*

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3. AMENDMENT HISTORY

Amendment No.	Protocol Version No.	Date issued	Author(s) of changes	Details of Changes made
1	1.2	10.04.2015	Sourij, Tripolt	Glucose monitoring device was added (MedTronic Ipro 2)
2	1.3	13.05.2015	Sourij, Tripolt	Bioelectrical impedance analysis (BIA) was added
3	1.4	03.07.2015	Sourij, Tripolt	Dry eye assessment, Ocular Surface Disease Index Questionnaire
4	1.5	22.09.2015	Sourij, Tripolt	Changes in inclusion criteria (body mass index:22.0-30.0kg/m^2)
5	1.6	10.02.2016	Sourij, Tripolt	FMD (Flow Mediated Dilatation) was added
6	1.7	13.03.2016	Sourij, Tripolt	Number of patients was reduced from 100 to 90.

4. SYNOPSIS

Study title	InterFast
Study design	Cohort study with an embedded randomized controlled pilot trial
Study participants	Healthy subjects and subjects who do already practice Alternate Day Fasting
Number of participants	90 (30 Participants in Alternate Day Fasting group and 60 participants in the control group). Those participants in the control group will be asked to participate in a short randomized controlled trial, where they will be either allocated to an Alternative Day Fasting group or another control visit.
Planned duration	18 months
Primary objective	The primary objective is to elucidate in which extent alternate day fasting influences human physiology in healthy individuals in both, the short and long term. Data of the pilot RCT will help to design a larger trial investigating Alternate Day Fasting in various groups of interest.
Secondary objectives	The secondary objective of this study is to define novel molecular markers of aging and age-related diseases.

5. BACKGROUND AND RATIONALE

Intermittent fasting is a dietary regimen defined by alternating fasting and "feeding" cycles. In addition to caloric restriction (a dietary regimen limited to a daily food intake lower than one's daily caloric needs) only, intermittent fasting seems to activate cell autophagy (cellular "recycling" program) which potentially increases cellular stress resistance and removes accumulated molecules that are potentially toxic[1-3]. In fact, mice maintained on intermittent fasting without decreased overall food intake show effects on body weight reduction that equal and in some cases even exceed those of calorie restriction[4, 5]. However, additionally, intermittent fasting combined with even a high-fat diet in the feeding periods protects mice from obesity, hyperinsulinemia, hepatic steatosis, and inflammation compared to controls that are fed an *ad libitum* high-fat diet despite the same calorie intake[6], making this intermittent fasting regimen a promising approach to reduce morbidity and mortality in various species.

The best described and most widely practiced version of intermittent fasting is the "alternate day diet" or "alternate day fasting" (ADF). In animal models, ADF consists of an ad libitum "feed day" alternated with a 100% restriction "fast day". However in humans, this is often modified to allow a small amount of food consumption on the "fast day" (e.g. 25% of the individual´s energy needs). Findings from recent modified ADF studies showed 4-8% reduction in body weight after 8-12 weeks[7-10]. This reduction in body weight is usually escorted by reductions in triglycerides[8, 10, 11], low density lipoprotein (LDL) cholesterol[8, 10, 11], systolic blood pressure[10], insulin resistance[7] and increases in LDL particle size[7, 12, 13].

However, knowledge about the molecular effects of the alternate day diet on human metabolism or autophagy is still scarce since detailed analyses of molecular and metabolic parameters remain unexplored, especially in healthy individuals. The overarching aim of this research project is to elucidate in which extent alternate day fasting (and thereby intermittent fasting) influences human physiology in healthy individuals in both short and long term. The secondary objective of this study is to define novel molecular markers of aging and age-related diseases.

6. AIMS OF THE STUDY

The overarching aim of our cohort study with embedded pilot randomized control trial is to investigate the effects of repeating fasting periods on human physiology, aging process and molecular cellular processes in humans. We will be able to study long term effects (subjects in the cohort study, who already practise ADF for a defined time period) and short term effects (subjects randomized to the ADF group) of this nutritional intervention.

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7. OBJECTIVES

7.1 Primary objective

The primary objective is to elucidate in which extent ADF (and thereby intermittent fasting) influences human physiology in healthy individuals in both short and long term.

7.2 Secondary objective

The secondary objective of this study is to investigate novel molecular markers and cellular pathways of aging and age-related diseases and whether they are influenced by ADF.

7.3 Long-term objective

The information gained in this project will be used to develop a strategy for follow-up studies in model organisms, such as yeast, worms, fruit flies, mice and humans. As a long term goal, we want to identify new targets (metabolites, macromolecules and pathways) that can be used for more precise clinical diagnosis and in the treatment and prevention of aging and age-related diseases. We will accomplish this by mimicking such nutritional regimen in animal models (methods are already well established in Prof. Madeo's or his collaborator's laboratories) in combination with knock-out and knock-down experiments, that will help differentiate between correlative and causal connection between nutrition, health and disease.

8. OUTCOMES

8.1 Primary outcome measures in the cohort study

- Differences in body composition (fat mass and lean body mass) between groups
- Differences in resting energy expenditure between groups
- Differences in insulin sensitivity (Matsuda Index, ISI, HOMA-Index, QUICKI) between groups
- Difference in glycaemic pattern (continuous glucose measurement) between the two groups
- Differences in endothelial function (Mean Intima media thickness, Reactive Hyperaemia Index, Stiffness Index, Aortic augmentation index) between groups
- Differences in mean 24h blood pressure between groups
- Differences in hormonal regulation (fertility, appetite hormones and others)

8.2 Secondary outcome measures in the cohort study

- Levels of different cell signaling and cell cycle proteins (p16, p21, p53, NCX, mTOR,

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Ras, Rb)

- Differences in parameters of cell death (apoptosis, stress resistance, mitochondrial damage) between groups
- Differences in telomere length, double strand breaks, chromosomal aberrations, heterochromatin between groups
- Differences in telomerase activity between groups
- Differences in endocrine parameters of fertility and nutritional regulation

8.3 Primary outcome measures in the randomized controlled trial

- Difference in the changes of insulin sensitivity (Matsuda index, ISI, HOMA-Index, QUICKI)
- Difference in the change of endothelial function

8.4 Secondary outcome measures in the randomized controlled trial

- Levels of different cell signalling and cell cycle proteins (p16, p21, p53, NCX, mTOR, Ras, Rb)
- Differences in parameters of cell death (apoptosis, stress resistance, mitochondrial damage) between groups
- Differences in telomere length, double strand breaks, chromosomal aberrations, heterochromatin between groups
- Differences in telomerase activity between groups

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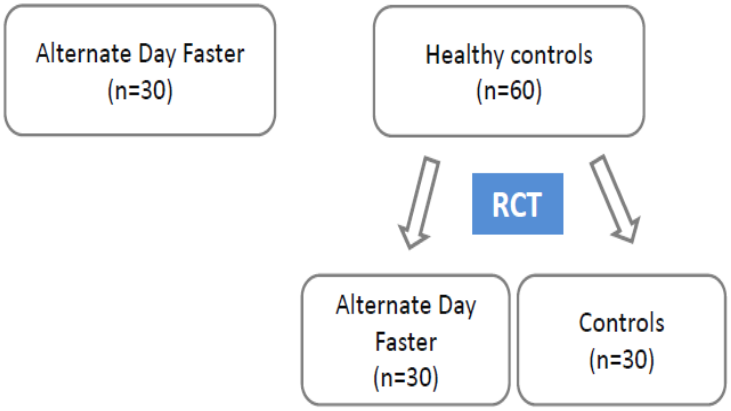
9. STUDY DESIGN

9.1 Schematic overview on study design



9.2 Duration of Study

It is anticipated that the study will run for 15 months (from first patient first visit until last patient last visit).

First patient first visit (FPFV):	May 2015
Last patient first visit (LPFV):	July 2016
Last patient last visit (LPLV):	August 2016
Data base lock:	September 2016
First results:	October 2016

9.3 Data collection

Once informed consent has been obtained, recruiting clinicians complete a CRF for each participant to record socio-demographic information, information about medical history and medications and dietary habits. Source documents comprise the CRF and hospital records, laboratory records and correspondence. All documents will be stored safely in a confidential manner. The subject will be referred to by a unique study subject number/code, their initials and date of birth on all study-specific documentations. The only exceptions will be the signed Consent Forms, Subject Identification log and subject clinical file, all of which will be stored securely by the clinical site. Source data will be made available for internal and external audits or inspections by regulatory authorities to authorised personnel only.

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9.4 Randomization

Randomization for the pilot RCT will be designed and performed by the "Randomizer tool" provided by the Institute of Medical Informatics, Statistics and Documentation of Medical University Graz, Austria.

10. SELECTION AND WITHDRAWAL OF STUDY SUBJECTS

10.1 Subject Selection

The study population will consist of 100 healthy subjects. Fifty participants of them should already practice Alternate Day Fasting. Subjects in the ADF and control group will at least be matched by sex, weight and age. Subjects of the control group will be asked to participate in a pilot randomized controlled trial. A separate informed consent will be used for the intervention study.

10.2 Subject Recruitment

Healthy controls will be identified via Primary Care and adverts. Alternate Day Fasting subjects will be recruited by adverts and leaflets at Alternate Day Faster Events.

10.3 Inclusion Criteria

- ✓ Age between 35 and 65 years,
- ✓ Body mass index in the range of 22.0 – 30.0 kg/m^2,
- ✓ Fasting blood glucose <110mg/dL (without medication)
- ✓ LDL-cholesterol <180 mg/dL (without medication)
- ✓ Blood pressure <140/90 mmHg (without medication)
- ✓ Stable weight (change <± 10%) for 3 months immediately prior to the study,
- ✓ No history of metabolic disorders or cardiovascular disease
- ✓ No acute or chronic inflammatory disorder
- ✓ No current medications to regulate blood sugar, blood pressure or lipids or hormones
- ✓ No heavy drinking (more than 15 drinks/week)
- ✓ No use of tobacco or recreational drugs within past 5 years
- ✓ No dietary restrictions (e.g. vegetarianism and vegan)

10.4 Exclusion Criteria

- ✓ Known Malignancy

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- ✓ Women who are pregnant, breast-feeding or trying to become pregnant
- ✓ History of any chronic disease process that could interfere with interpretation of study results
- ✓ Women or men on hormonal supplementation or anti-conceptive hormonal medication for at least 2 months
- ✓ Therapy with antidepressants within past 6 months
- ✓ Regular therapy with acetylsalicylic acid

10.5 Withdrawal / Drop out of subjects

Each subject has the right to withdraw from the study at any time without prejudice or compromise to future care.

The investigator may discontinue or withdraw the subjects under the following circumstances:

- Significant protocol deviation
- Significant non-compliance with treatment regime or study procedures
- An adverse event that requires discontinuation of the study medication or results in inability to continue to comply with study procedures. If a subject is withdrawn due to an adverse event, the investigator will arrange for follow-up visits or telephone calls until the adverse event has resolved or stabilised.
- Consent withdrawn
- Lost to follow up
- Any other situation that may, in the opinion of the investigator, make it unsafe or inappropriate for the subject to continue in the trial

10.6 Payment to subjects

Reimbursement of reasonable travel expenses will be considered.

11. STUDY PROCEDURES

11.1 Schedule of study procedures of the cohort study

	Visit 1	**Visit 2** (9 ± 4 days after visit 1)
Informed Consent	X	
Inclusion/exclusion criteria	X	
Demography, medical history	X	

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Concomitant medication	X	X
Vital signs	X	X
Physical examination	X	
Blood sampling (FMD)	X	X
Dual-energy X-ray absorptiometry (DXA)		X
Oral glucose tolerance test (OGTT)	X	X (ADF group only)
Electrocardiography (ECG)	X	
Questionnaires (FFQ, Physical Activity)		X
Retinal vessels analyser		X
Continuous Glucose monitoring (CGM)	X	
Hand-Grip Test		X
Accelerometer (Movisens®)	X	
Endothelial function (EndoPat 2000)		X
Intima media thickness (IMT)		X
Pulse wave analysis		X
Buccal mucosa sampling	X	
24h blood pressure measurement	X	
Sputum sampling	X	
Faeces sampling	X	
Echocardiography	X	
Resting energy expenditure (REE)		X
Bioelectrical impedance analysis (BIA)		X
Dry eye assessment (optional)		X
Flow mediated dilatation (FMD)		X

11.2 Visit Schedule for the cohort study

Visit 1

- Sufficient time will be provided and subjects will be encouraged to discuss all queries with the research team
- Informed consent for the study will be obtained by an investigator
- Biobank informed consent will be obtained by an investigator
- Check inclusion and exclusion criteria

- Demographics will be obtained: date of birth, gender, race, smoking status, alcohol consumption
- Weight
- Height
- Waist-Hip Ratio
- Medical history and standardized questionnaire on fertility outcomes
- Concomitant medication
- Vital signs: resting pulse and blood pressure
- Blood sampling
- ECG
- Food Frequency Questionnaire and Ferriman-Galway Score/Hamilton-Norwood Scheme
- Baseline blood tests (fasting): Full blood cell count, renal function tests, liver function tests,
- Pregnancy test in female woman of childbearing age
- Oral glucose tolerance test (OGTT)
- Application of the 24h blood pressure device
- Application of continuous glucose monitoring
- Application of accelerometer (Movisens®)

Visit 2 (9 ± 4 days after visit 1)

- Vital signs, Weight
- Physical examination
- Concomitant medication review
- Resting energy expenditure (REE)
- Measurement of endothelial function (EndoPAT 2000)
- Flow mediated dilatation (FMD)
- Blood sampling
- Oral glucose tolerance test (OGTT)
- IMT measurement
- Retinal vessel analyser
- Hand Grip Test
- Pulse wave analysis
- Echocardiography
- Collection and analysis of 24h blood pressure monitor
- Collection and analysis of accelerometer

- Removal of continuous glucose monitoring
- Dual-energy X-ray absorptiometry (DXA) and hand grip test
- Collection of sputum and faeces samples
- Buccal mucosa sampling
- Informed consent for RCT (healthy control group willing to participate in the RCT)
- Bioelectrical impedance analysis (BIA)
- Dry eye assessment (optional)

Visit 3 (2 weeks before visit 4) → only those subjects participating in the pilot RCT

- Application of continuous glucose monitoring

Visit 4 (4 weeks ± 1 week after visit 2) → only those subjects participating in the pilot RCT

- Vital signs, Weight
- Physical examination
- Concomitant medication review
- Resting energy expenditure (REE)
- Measurement of endothelial function (EndoPAT 2000)
- Flow mediated dilatation (FMD)
- Blood sampling
- Food Frequency Questionnaire
- Oral glucose tolerance test (OGTT)
- Removal of continuous glucose monitoring
- Collection of sputum and faeces samples
- Buccal mucosa sampling
- Bioelectrical impedance analysis (BIA)

Visit 5 (2years ± 4 weeks after Visit 4) → → only those subjects participating in the pilot RCT

- Vital signs, Weight
- Physical examination
- Concomitant medication review
- Blood sampling

- Food Frequency Questionnaire

11.3 Schedule of study procedures of the pilot intervention trial

	Visit 3 (14 ± 2 days after visit 2)	**Visit 4** (4 weeks ± 1 week after visit 2)	**Visit 5** (**2years** ± 4 weeks after Visit 4)
Application of continuous glucose monitoring	X		
Concomitant medication		X	X
Vital signs		X	X
Physical examination		X	X
Blood sampling		X	X
Oral glucose tolerance test (OGTT)		X	
Continuous Glucose monitoring (CGM)		X	
Endothelial function (EndoPat 2000)		X	
Buccal mucosa sampling		X	
Saliva sampling		X	
Faeces sampling		X	
Resting energy expenditure (REE)		X	
Questionnaires (FFQ, Physical Activity)		X	X
Bioelectrical impedance analysis (BIA)		X	
Flow mediated dilatation (FMD)		X	

11.4 Laboratory results

All laboratory results will be reviewed and the reports signed by the study physician who will record whether it is normal, abnormal but not clinically significant, or abnormal and clinically significant. In the latter case, the eligibility of the subject will be reviewed.

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12. METHODS

12.1 Oral glucose tolerance test (OGTT)

Various insulin sensitivity indices derived from a frequently sampled, extended oral glucose tolerance test (OGTT) will be calculated accounting for the various weighting of hepatic and peripheral insulin sensitivity expressed by the different indices. Insulin, C-peptide and glucose will be measured before and 15, 30, 60 and 120 minutes after ingestion of 75g glucose (®Glucoral 75 citron, Germania Pharmazeutika, Vienna). In addition, lipid-parameters (total cholesterol, triglycerides, HDL, LDL, free fatty acids) and a routine safety lab will be measured. The OGTT will be performed after an overnight fast (apart from water). A standard gauge cannula will be placed into a subcutaneous vein for blood sampling. In order to prevent blood clotting in the cannula and to keep the cannula working it will be occasionally flushed with sterile normal saline. A pre-meal blood sample will be taken (-5 mins) and then all subjects will be asked to drink the sugar solution over a period of 2-4 mins (time 0 min). During the OGTT further blood samples will be taken at 15, 30, 60, and 120 minutes. The blood at each time point will be placed into a fluoride oxalate tube (1ml) for plasma glucose and into a serum tube for analyses of insulin and c-peptide.

The insulin sensitivity index will be calculated from the OGTT according to four different equations: the Matsuda-Index (IS_{OGTT}): $ISoGTT = \frac{10000}{\sqrt{(glucose0 * insulin0) * (mean\ glucose * mean\ insulin)}}$, [14] the HOMA-IR (Homeostasis Model Assessment for Insulin Resistance): $HOMA - IR = \frac{FPG(^{mmol}/_{l}) * FSI(^{U}/_{l})}{22.5}$ [15] and the $QUICKI = \frac{1}{\log(insulin0) + \log(glucose0)}$ [16]. The last equation was proposed by Stumvoll et al. (insulin sensitivity index ISI): $ISI = 0.222 - 0.00333 * BMI - 0.0000779 * Ins120 - 0.000422 * age$ 17.

Beta-cell function will be estimated in the fasting state with $HOMA - \beta = \frac{20 * Insulin0}{Glucose0 - 3.5}$ 15 and during the OGTT with the Stumvoll-Index: $1st\ phase = 1283 + 1.829 * Ins30 - 138.7 * Glc30 + 3.772 * Ins0 \quad plus \quad 2ndphase = 286 + 0.416 * Ins30 - 25.94 * Gluc30 + 0.926 * Ins0$ 17 and the ratio of the incremental insulin to glucose response over the first 30min during the OGTT $\frac{\Delta\ Insulin\ (30)}{\Delta\ Glucose\ (30)}$.

12.2 Endothelial function

Endo-PAT 2000 (Itamar Medical Ltd., Caesarea, Israel) will be used to measure endothelium-dependent vaso-reactivity as previously described [18]. In brief, before measurements, the subjects will be in supine position for a minimum of 10 minutes, in a quiet, temperature-controlled room with dimmed lights.

Probes will be placed on both index fingers and pulse wave amplitudes will be detected and recorded during the study. After a five-minute baseline measurement, arterial flow will be occluded using a cuff on the non-dominant arm. The cuff will be inflated to 60 mmHg above systolic pressure. After five minutes of occlusion, the cuff will be rapidly deflated to allow reactive hyperemia. Pulse wave amplitudes will be recorded again for at least five minutes. The ratio of the peripheral arterial tone (PAT) signal after cuff release compared with baseline will be calculated through a computer algorithm automatically normalizing for baseline signal and indexed to the contra lateral arm. The calculated ratio represents the reactive hyperemia index (RHI) [19].

12.3 Bone densitometry and body composition

Bone density scanning, also called dual-energy x-ray absorptiometry (DXA) or bone densitometry, is an enhanced form of x-ray technology that is used to measure bone loss. DXA measurement will be performed with a GE Lunar iDEXA (GE Healthcare, Waukesha, WI, US) full size for the purpose of estimating percentage body fat according to the departmental Standard Operating Procedure. Body regions are defined using standard anatomical partitions. Scan areas are analysed to determine lean mass, fat mass, bone mineral content, and total body composition.

12.4 Laboratory measurements (Clinical and hormonal)

Insulin and c-peptide will be measured by chemiluminescence on an ADVIA Centaur system (Siemens Healthcare Diagnostics, Eschborn, Germany). Fertility hormones (AMH (Anti-muellerian hormone), testosterone, cortisol, thyreotropin, triiodothyronine and thyroxine, estrogen, SHBG (sexual hormone binding globulin), LH (luteotropic hormone), FSH (follicle stimulating hormone) and 25(OH)vitamin D will be measured using automated analyzers: AMH by Beckmann-Coulter, Krefeld, Germany; testosterone, cortisol, thyreotropin, triiodothyronine and thyroxine by Siemens ADVIA Centaur, Eschborn, Germany; SHBG by Roche Diagnotics, Mannheim, Germany; estrogen, LH and FSH by Triturus, Biomedical Diagnostics, Antwerp, Belgium, 25(OH)vitamin D by iSYS, IDS, Boldon, U.K.) respectively. Samples for appetite hormones (Leptin, Ghrelin, PYY, and others) will be stored at -80°C until analysis after centrifugation. In addition, lactose intolerance (as defined by the locus LCT-13910) and vitamin D genotypes will be determined by standardized PCR from EDTA blood samples. FFA will be measured enzymatically (Wako Chemical, Neuss, Germany) on an Olympus AU640 (Olympus Diagnostica, Hamburg, Germany). Routine parameters will be determined using a cobas® analyzer (Roche Diagnostics, Mannheim, Germany).

12.5 Laboratory measurements (Specialized Research – Aging Parameters)

Molecular parameters of aging will be measured by a variety of already established methods at the Prof. Madeo's laboratory at the Institute of Molecular Biosciences, University of Graz. Levels of different cell signaling and cell cycle proteins (p16, p21, p53, NCX, mTOR, Ras, Rb) will be measured using capillary-based western blot system – Wes™ (Proteinsimple, San Jose, California, USA). Inflammatory markers, insulin signaling, as well as precise characteristics of molecular pathways will be analyzed using certified and commercially available V-PLEX Assays (Meso Scale Discovery, Rockville, Maryland, USA). Epigenetic changes (methylation and acetylation) will be analyzed via traditional western blotting. All methods based on antigen-antibody interaction will be done using commercially available antibodies, in order to assure better reproduction and optimal processes and procedures. Different "omics" (metabolomics, proteomics, acetylproteomics) as well as levels of different polyamines will be measured using HPLC-coupled mass spectrometry (MS) or HPLC-coupled MS/MS methods. Different parameters of cell death (apoptosis, stress resistance, mitochondrial damage) and damage caused by aging (telomere length, double strand breaks, chromosomal aberrations, heterochromatin changes) will be analyzed using flow cytometry systems BD Bioscience LSRFortessa and FACSAria IIu at the NAWI Graz Central Lab "Graz Cell Informatics and Analyses". The telomerase activity will be measured by Human Telomerase Reverse Transcriptase (hTERT) ELISA Quantitation Kit (GenWay, San Diego, California, USA).

12.6 Physical activity measurement

The MoviSens-device consists of a 5.3×3×2-cm-sized body and can be fixed with a clip to the hip. During the measurements, the sensor will be placed on the right side of the subjects' hip. The sensor is a three-axial acceleration sensor with a range of ±8 g, a resolution of 12 bit and a sampling rate of 128 Hz. The recorded data from the sensor, including raw data from the acceleration sensor, can be displayed on a computer when connected to it via USB cable. Energy expenditure is displayed in steps of 1 s. Short time intervals allow monitoring spontaneous activities. The recognition of different activities is based on the extraction of mathematical and statistical features of the raw acceleration signal. The features are calculated for each segment of 4 s. Calculated features are, amongst others, maximum frequency, step count, and the number of mean crossings. These features are the input information of a decision tree which classifies the activity of the person. Activities that can be detected are rest (combination of lying, sitting, and standing); bicycle or ergometer; going upstairs; walking (combination of jogging, going downstairs, walking slow, normal, and fast); and unknown activity. According to information from the manufacturer, the decision tree was generated using data of approximately 300 subjects who performed

daily life activities. The accuracy of the activity recognition algorithm is discussed by Jatobá et al. [20]. According to the detected activity, one of five different models is selected.

12.7 Intima media thickness (IMT)

The measurement of the common carotid artery intima and media combined layers as determined by high-resolution B-mode ultrasonography is widely recognized as a useful tool for early identification for systemic atherosclerosis. Intima media thickness (IMT) and plaque will be measured using high resolution ultrasound. IMT will be measured in the common carotid artery in a segment of 2 cm below the bulb. Near and far wall measurements of both sides will be combined to calculate mean IMT [21]. Plaque burden will be quantified using the B-score according to the ACAPS protocol [22].

12.8 Food frequency questionnaire (FFQ) and hormonal scores

A self-administered, semi-quantitative FFQ was developed to assess usual food consumption within the German Health Examination Survey for Adults 2008-2011 (DEGS) [23]. The relative validity of this questionnaire was studied among participants of another nationwide survey, the German National Nutrition Monitoring (NEMONIT) [24]. The FFQ includes questions about the frequency and the amount of 53 food items, consumed during the past four weeks. Frequency of consumption of food items was asked according to specified categories. In addition, the respondents had to indicate the portion size of the food items consumed in predefined answering categories. Pictures were used to aid the estimation of portion size for 33 food items. In total, 29 food groups will be presented by the food frequency questionnaire. Standardized fertility questionnaires are published medical history questions asking for menarche/menopause, menses regularity and childbirth/child wish in female and complementary data in male probands. Ferriman-Galway Score and Hamilton-Norwood Scheme are standardized questionnaires/schemes for female hirsutism or male baldness, respectively. Probands and/or medical doctors use pictograms for a scoring of female/male body hair.

12.9 Non-invasive 24h ambulatory blood pressure monitoring

Subjects wear the blood pressure device for a single 24-hours period. During this time, the device is programmed to inflate and record blood pressure at pre-specified intervals (usually every 15min during daytime hours and every 30 minutes during night-time hours), which provides approximately 50-75 blood pressure recordings during the 24-hours period. Study participants are asked to keep an activity log throughout the 24-hour period so activities can mapped onto the blood pressure recordings. The process for educating subjects about the blood pressure device and setting up the test takes

approximately 10 minutes. Patients should undergo testing on a regular workday. They should be educated about the process of monitoring and the importance of maintaining an activity log.

12.10 Resting energy expenditure (REE)

The resting energy expenditure (REE) is a component of energy expenditure that is measured by indirect calorimetry (IC). Subjects must at least 30-minute rest after at least 8 hours of sleep and after at least 3 hour fasting. During measurement, subject must be kept fully awake, lied down quietly, completely relaxed and breathing normally. The measurements took place for at least 30 minutes and were performed in standard neutral hospital room temperature. A breath mask will be placed over the head of the subject. Oxygen consumption and carbon dioxide production will be measured and energy expenditure will be calculated by the Weir formula [25].

12.11 Hand Grip Test

One of the most common methods of measuring muscle strength is the isometric grip strength test. We will measure isometric grip strength using a handgrip dynamometer (JAMAR®, Nottinghamshire, UK). The test will be performed in the sitting position with the shoulder of tested arm adducted, the elbow flexed at 90°, whereas the forearm and wrist were set in neutral position [26]. The testing protocol consists of three maximal isometric contractions for 5 s, on both hands, with a rest period of at least 60 s and the highest value will be used for determination of maximal grip strength. The subjects will be instructed to squeeze the dynamometer as hard as possible.

12.12 Pulse wave analysis

Radial Pulse wave analysis (PWA) and carotid-femoral pulse wave velocity (PWV) will be performed using the Sphygmocor tonometry system (AtCor Medical, Sydney, Australia) and assessed as previously described[27]. Participants will be in the supine position. Briefly, a central aortic pressure waveform will be derived from a measured radial artery pressure waveform. Aortic augmentation index (AIx) will be calculated as the difference between the first and second systolic peaks of the ascending aortic waveform divided by the difference between central aortic systolic blood pressure (SBP) and central aortic diastolic blood pressure (DBP). AIx will be standardized to a heart rate of 75 beats/minute (AIx75). To determine PWV, ECG-gated pulse pressure waveforms will be obtained sequentially over the right carotid artery and the right femoral artery. PWV will be calculated as the transit distance between the measurement

sites divided by the transit time computed as the time interval between the feet of the two pressure waves.

12.13 Echocardiogram (ECG)

A 12-lead ECG will be performed by a co-investigator or delegated staff. The ECG print out must be interpreted, signed and dated by a co-investigator.

12.14 Buccal mucosa sampling

Perform mouth wash with distilled water (three replicates) to remove traces of saliva and surface mucous. Then perform exfoliation within the two cheeks to maximize the cell sample and remove any trace unknown, which may be caused by sampling of only one cheek. The exfoliation should use a disposable brush similar to that used for Pap smear, performing 10 rotations of the brush against the inner wall of the cheeks starting in the centre and gradually increasing the circumference, producing a spiral effect to increase the sampling of a larger area and prevent erosion continuous of a single region. After exfoliation, the brushhead must be placed in a container (test tube) with 04ml of buffer containing: 0.01 M Tris hydrochloride (Tris HCl), 0.1 M acid ethylenediaminetetraacetic (EDTA) and 0.02M Sodium Chloride (NaCl) with pH 6.8, turning in the way that the cells are released and displaced on the inner edge of the container [28].

The micronucleus (MN) assay in exfoliated buccal cells is a useful and minimally invasive method for monitoring genetic damage in humans [29]. The Buccal Micronucleus Cytome (BMCyt) assay is a minimally invasive method for studying DNA damage, chromosomal instability, cell death and the regenerative potential of human buccal mucosal tissue. This method is increasingly used in molecular epidemiological studies for investigating the impact of nutrition, lifestyle factors, genotoxin exposure and genotype on DNA damage, chromosome malsegregation and cell death. The biomarkers measured in this assay have been associated with increased risk of accelerated ageing, cancer and neurodegenerative diseases [30].

12.15 Blood sampling and storage

Over the study in those participating in the cohort study only in total approximately 150 ml of blood will be taken to determine the parameters outlined above, in those also participating in the RCT in total (cohort study and RCT) approximately 200 ml of blood will be drawn. A potential remainder of serum and plasma will be stored anonymised (patient number and visit number only) at -80°C in the Biobank

of the Medical University of Graz for potential future analyses. Therefore subjects will be asked to sign the informed consent of Biobank.

12.16 Faeces sampling

Sampling will be performed using two stool collection tubes by Sarstedt, Nümbrecht, Germany, using a stool collector (Süsse Stuhlfänger, Gudensberg, Germany). One tube will be used for native stool, the other prefilled with stool DNA-stabilizer for collection of DNA-stabilized stool specimen. Directions for safe and hygienic fecal collection will be delivered to the probands in words as well as in pictograms.
Bacterial DNA will be extracted from saliva and stool samples using the MagNA Pure LC DNA Isolation Kit III (Roche). The 16S rRNA gene will be amplified in a PCR reaction and sequenced with next-generation sequencing technology (Roche Genome Sequencer FLX or Illumina MiSeq) and interpreted by the respective software analyzers.

12.17 Saliva sampling

Saliva will be collected by Salitubes (DRG Instruments Marburg, Germany) using a routine method for saliva collection and dedicated tubes as well as standardized instructions for the collection. Hormones and salivary microbiome will be analysed by methods described in 12.4 (adapted ELISAs by BSM, Vienna, Austria) and 12.16, respectively.

12.18 Continuous glucose monitoring

Minimally invasive CGMS will be performed using the Abbott FreeStyle Flash or Medtronic Ipro 2 glucose monitoring device. This system eliminates the need for routine finger pricks, reading glucose levels through a sensor that can be worn on the back of the upper arm for up to 7-14 days. In addition, no finger prick calibration is needed—a key differentiator from current continuous glucose monitoring systems. Both, Abbott´s FreeStyle Flash and Medtronic Ipro 2 glucose monitoring systems, consist of a small, round sensor—approximately the size of a two Euro coin—worn on the back of the upper arm, which measures glucose every minute in interstitial fluid through a small (5mm long, 0.4mm wide) filament that is inserted just under the skin and held in place with a small adhesive pad. A reader is scanned over the sensor to get a glucose result painlessly in less than one second. Scanning can take place while the sensor is under clothing, making testing more discreet and convenient. Each scan displays a real-time glucose result, a historical trend and the direction the glucose is heading. The reader holds up to 90 days of data, providing a historical snapshot of glucose levels over time. The Medtronic devices

requires regular (at least twice daily) calibration measurements with a common glucose measurement device.

In the cohort study the CGM sensor will be used once (14 days, at visit 1) and in those subjects participating in the pilot intervention trial, CGM will be used a second time (another 14 days, CGM will be started at visit 3).

12.19 Physical examination

Physical examination will be performed at visit 1 and include examination of:

- General appearance
- Head, ears, eyes, nose, throat, neck
- Cardiovascular system
- Respiratory system
- Skin
- Thyroid gland
- Musculoskeletal system
- Central and peripheral nervous system

12.20 Vital signs

Systolic and diastolic blood pressure measured in mmHg will be collected at both visits to the trial site. The blood pressure should be measured in a sitting position, with legs uncrossed, the back and arms supported. Subjects should be sitting for at least five minutes before the measurement is taken and subjects should not talk during the measurement. Pulse as beats per minute will be recorded after resting for five minutes in a sitting position. The pulse should be measured at both study visits to the trial site.

12.21 Echocardiography

Echocardiographic examination will be performed with a Vivid 7 (GE Healthcare, Chalfont St Giles, UK). For acquisition of images in the parasternal view the participant will be placed in the steep left-lateral decubitus position. The participant's left arm will be raised. Images will be acquired during quiet respiration. Image rate will be at least 30 frames per second.

12.22 International physical activity questionnaire (IPAQ)

IPAQ will be used in the current study. This tool was developed for measuring physical activity in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites)

during 2000[31, 32]. The final results suggested that this tool had acceptable measurement properties for use in many settings and in different languages and was suitable for national population-based prevalence studies of participation in physical activity. The short version of IPAQ tool will be used in the current study. The tool includes a set of 4 questionnaires. The questions asked about the time spent being physically active in the last 7 days.

12.23 Bioelectrical impedance analysis (BIA)

Bioelectrical impedance analysis is a fast, reliable, affordable and non-evasive technique to measure body composition. Its accuracy and dependability have been scientifically proven [33, 34] and clinically demonstrated time and time again. It uses the fact that tissue types differ in electric conductivity. Bones and fat, for instance are bad conductors. Skeletal muscles on the other hand, due to their water and electrolyte content, have much lower impedance. BIA makes it possible to precisely determine the distribution of the individual components, e.g. fluids ("water"), muscle mass and fatty substance in the body.

12.24 Dry eye assessment

Recent findings show that dry eyes syndrome ("Sicca" syndrome) is tightly regulated by the metabolic and inflammatory factors both on the eye surface and on a systemic level during aging [35]. Since we propose intermittent fasting besides caloric restriction [36] as one of the most significant interventions into the human metabolism, we will conduct the preliminary evaluation of the effects of intermittent fasting on several main clinical parameters of the Sicca syndrome. As part of our trial we will investigate:

- redness of cornea and the stability of basal tear film by using Oculus Keratograph 5M
- the degree of epithelial damage on the eye surface by fluorescein staining
- the tear volume by Schirmer's test I
- the quantity and the quality of the meibomian gland's secretion
- the metabolome of basal tear fluid by mass spectrometry
- the risk factors and symptoms of the Sicca syndrome by using the OSDI (Occular Surface Diseases Index) questionnaire.

All procedures are carried out under non-invasive conditions and lead to no influence of the eye function of the participant, both in short and long term. By analysing the effects of intermittent fasting on the development of dry eyes syndrome, we want to examine the potential of such a treatment as a preventive and possibly even interventional method to sustain and improve eye health during aging.

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12.25 Flow mediated dilatation (FMD)

Endothelium-dependent FMD following reactive hyperaemia is examined in the brachial artery according to the guidelines described by Corretti et al [37]. Using high resolution ultrasound measurements of the right brachial artery are taken after resting and after cuff deflation (250 mmHg for 5 minutes) of the forearm. Scans of the brachial artery are taken proximal to the antecubital fossa with simultaneous ECG recording. Diameter measurements are taken from one media-adventitia interface to the other for three to four times at baseline (the average is taken for calculating the baseline diameter) and three times after 45 to 60 seconds after cuff deflation following reactive hyperaemia. FMD-diameter is calculated as the average of the three diameter measurements following reactive hyperaemia. The dilatation is calculated as the percent change in diameter compared to baseline.

12.26 Data analysis and sample size estimation

Data analysis

Statistical Analyses for primary and secondary outcomes as well as for baseline values are done in a descriptive and exploratory way.

Continuous variables will be summarized with standard descriptive statistics tables and represented graphically with displays such as box plots and histograms, and categorical variables will be described by frequency tables and standard graphical displays like bar plots. Scatterplots are used to illustrate possible correlations between the variables.

To examine differences between groups at baseline and following visits, Student's T-tests or Mann-Whitney U tests and chi-square tests will be used as appropriate. Comparisons within groups will be performed using paired T-test or Wilcoxon rank-sum test. A p-value of <0.05 is considered to indicate statistical significance, whereas appropriate correction for multiple testing like Bonferroni Correction or false discovery rate (*Benjamini* and *Hochberg*) will be applied. To investigate multiple relations generalized linear mixed models will be built to investigate changes between groups and over time, as the physiological changes are represented in various parameters. For high-dimensional "omics-" data appropriate multivariate modelling like Random Forests and Partial Least Squares Regression will be used to elucidate differences between groups and time-points concerning potential molecular markers regarding aging effects.

A statistical analysis plan will be prepared and finalized before database lock. All statistical analysis will be done in R (version 3.0.1.).

Sample size estimation

Data on the effects of ADF on metabolic parameters, human physiology, aging process and molecular cellular processes in healthy subjects is very limited and a therefore an accurate sample size estimation impossible. However, we reviewed available data and used data on insulin sensitivity from a similar, albeit not entirely comparable study population with a HOMA-IR of 3 ± 1.1 (mean ± SD) [7]. Assuming a power of 80% and a type I error rate of 5%, we would need 50 subjects per group to demonstrate an effect size of 15%, which has been demonstrated previously [38]. Therefore the sample size of 50 subjects in the ADF and 50 subjects in the control group, as chosen for our cohort study, seems to be reasonable. In addition, previous publications have also suggested a sample size of 50 subjects per group for such types of studies with insufficient details about the outcome parameters required for a sample size estimation[39]. Subjects in the control group will be asked to participate in a pilot, randomized controlled trial, where they are randomized to either ADF or continuing in the control group. We expect an acceptance rate of about 40%, hence a sample size of about 10 participants per group.

12.27 Markers of cardiovascular risk

Serum Aliquots of 40 subjects will be used for miRNA-analyses (atherosclerosis-related intracellular signals) regarding cardiovascular risk between Japanese and Austrian cohorts (in cooperation with Ichiro Wakabayashi, MD PhD; Hyogo College of Medicine; Japan).

13. STUDY MONITORING

A risk-based approach will be taken to determine the frequency of study monitoring for the study which will be agreed with the Principal Investigator. Monitoring will be undertaken according to ICH GCP and the study monitoring plan. The study monitor will be suitably trained, qualified and experienced to

perform this task. Data will be evaluated for compliance with the protocol and accuracy in relation to source documents.

14. ETHICS

The Investigator will ensure that this study is conducted in accordance with the principles of the Declaration of Helsinki, GCP-ICH and according to the protocol and the requirements of the concerned regulatory authorities. The measurements taken are either non-invasive or minimally invasive (blood sampling). Therefore we consider this trial as low risk for participants.

14.1 Informed Consent

- 1. Informed consent will be obtained for all participants in the study by the study physician.
- 2. Written versions of the subject information and Informed consent will be presented to the subjects detailing:
    - The exact nature of the study
    - The implications and constraints of the protocol
    - The known side effects and any risks involved in taking part.
- 3. It will be clearly stated that the subject is free to discontinue their participation in the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal.
- 4. The subject will be allowed as much time as wished to consider the information, and the opportunity to question the Investigator, their usual care provider or other independent parties to decide whether they will participate in the study. Written Informed Consent will then be obtained by means of subject dated signature and dated signature of the person who presented and obtained the informed consent.
- 5. A copy of the signed Informed Consent will be given to the subjects. The original signed form will be retained at the study site.
-
- Every subjects participating in the cohort study will need to sign the cohort study informed consent and in addition the biobank informed consent. Those subjects willing to participate in the pilot RCT will be asked to sign an RCT specific informed consent in addition.

14.2 Ethical and Regulatory Approvals

The protocol, informed consent form, participant information sheet and any proposed advertising material will be submitted to the Ethics Committee of the Medical University of Graz, Austria.

The Investigator will submit and, where necessary, obtain approval from the above parties for all substantial amendments to the original approved documents.

15. FINANCE

This study will be jointly funded by the Institute of Molecular Biosciences, University of Graz and the Division of Endocrinology, Medical University of Graz, Austria.

16. INSURANCE

Participant insurance according to legal requirements will be contracted.

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