

Effect of the mitophagy inducer urolithin A on age-related immune decline: a randomized, placebo-controlled trial

In the format provided by the
authors and unedited

Supplementary Table 1

Specificity	Flourochrome	Clone	Vendor	Catalog number
BCL6	APC-H7	K112-91	BD_Biosciences	563581
Blimp-1	BV421	6D3	BD_Biosciences	565276
CD11c	eFluor_450	3.9	Thermo_Fisher	48-0116-42
CD123	Super_Bright_436	6H6	Thermo_Fisher	62-1239-42
CD127	APC-R700 (=AF700)	HIL-7R-M21	BD_Biosciences	565185
CD14	BUV615	M5E2	BD_Biosciences	751150
CD16	BUV496	3G8	BD_Biosciences	612944
CD19	Spark_NIR_685	HIB19	BioLegend	302270
CD27	PE-Fire_810	O323	Biolegend	302859
CD279	BUV661	EH12.1	BD_Biosciences	750260
CD3	PE-Alexa_Fluor_700	7D6	Thermo_Fisher	MHCD0324
CD4	cFluor_YG584	SK3	CYTEK	R7-20041-100T
CD45	PerCP	2D1	BioLegend	368506
CD45RA	BUV395	5H9	BD_Biosciences	740315
CD56	BUV737	NCAM16.2	BD_Biosciences	564447
CD8	BUV805	SK1	BD_Biosciences	612889
Eomes	PE-Cy7	WD1928	Thermo_Fisher	25-4877-42
FOXP3	PE-Dazzle_594	206D	Biolegend	320126
GATA3	BV711	L50-823	BD_Biosciences	565449
HLA-DR	BV750	L243	Biolegend	307671
IgD	BV480	IA6-2	BD_Biosciences	566138
IgM	BV570	MHM-88	BioLegend	314517
ki-67	BV605	Ki-67	BioLegend	350522
P16	AF647	EPR1473	Ab cam	ab192054
P21	AF488	EPR362	Ab cam	ab282187
Pax5	BV510	1H9	BD_Biosciences	563191
RORg	BV650	Q21-559	BD_Biosciences	563424
T-Bet	PerCP-Cy5.5	4B10	Biolegend	644805
TCRγδ	PerCP-eFluor_710	B1.1	Thermo_Fisher	46-9959-42
Tox	PE	TXRX10	Thermo_Fisher	12-6502-82
Viability	LIVE/DEAD_Blue	NA	Thermo_Fisher	L34962

Supplementary Table 1.

Antibodies used to identify human immune populations via spectral flow cytometry. Transcription factor panel is shown.

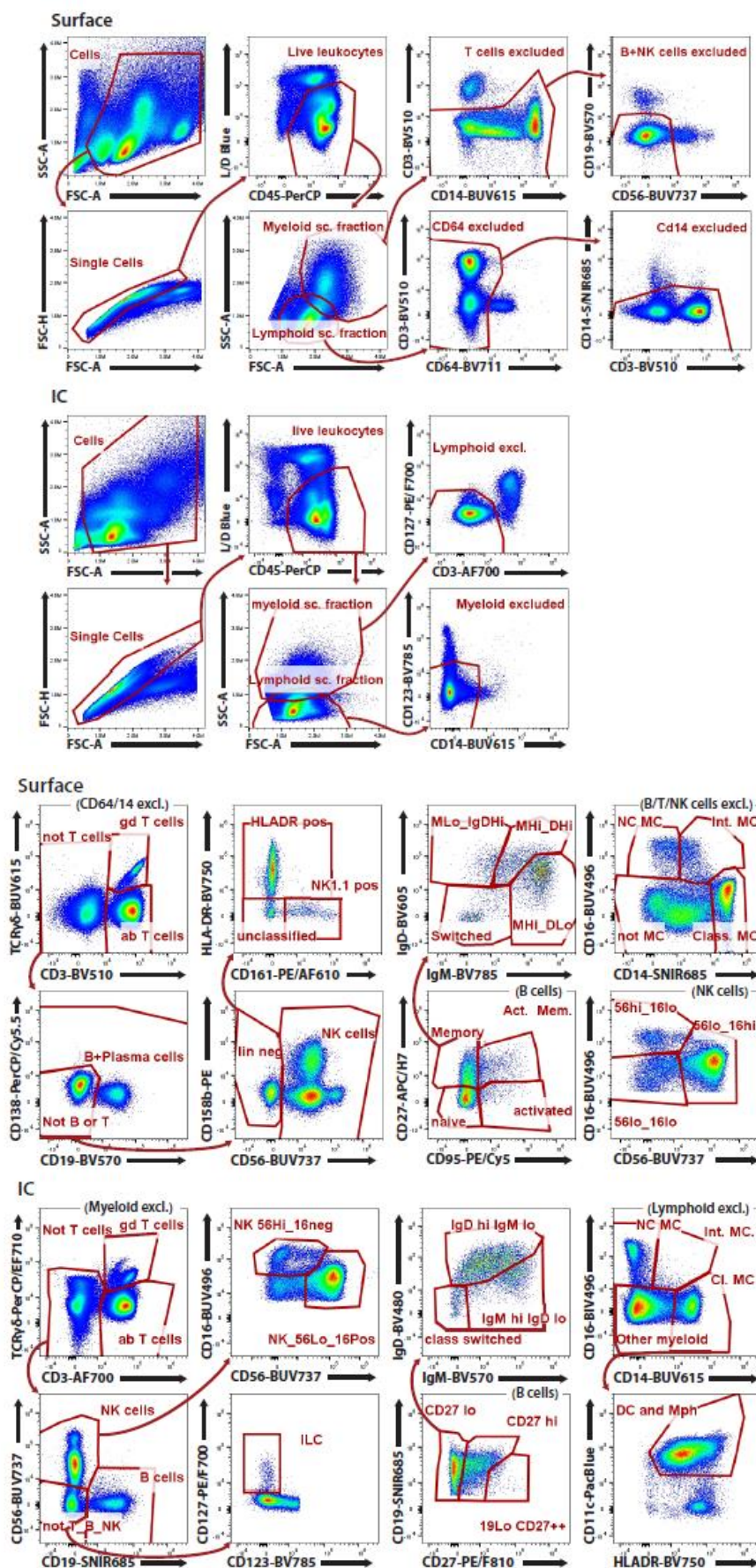
Supplementary Table 2

Specificity	Flourochrome	Clone	Vendor	Catalog number
C12FDG	FITC	NA	Thermofisher	D2893
CCR7	BV421	G043H7	BioLegend	353208
CD127	Super_Bright_436	eBioRDR5	Thermo_Fisher	62-1278-42
CD14	Spark_NIR_685	63D3	Biolegend	367150
CD158b	PE	DX27	Biolegend	312707
CD159a	AF700	S19004C	BioLegend	375119
CD16	BUV496	3G8	BD_Biosciences	612944
CD161	PE-Alexa_Fluor_610	HP-3G10	Thermo_Fisher	61-1619-42
CD19	BV570	HIB19	Biolegend	302236
CD25	PE-Alexa_Fluor_700	CD25-3G10	Thermo_Fisher	MHCD2524
CD27	APC-H7	M-T271	BD_Biosciences	560222
CD28	BV650	CD28.2	BioLegend	302945
CD3	BV510	SK7	BioLegend	344828
CD38	APC-Fire_810	HIT2	BioLegend	356643
CD4	cFluor_YG584	SK3	CYTEK	R7-20041-100T
CD45	PerCP	2D1	BioLegend	368506
CD45RA	BUV395	5H9	BD_Biosciences	740315
CD56	BUV737	NCAM16.2	BD_Biosciences	564447
CD57	eFluor_450	TB01	Thermo_Fisher	48-0577-42
CD8	BUV805	SK1	BD_Biosciences	612889
CD95	PE-Cy5	DX2	BioLegend	305610
HLA-DR	BV750	L243	BioLegend	307672
Klrg1	PE-Fire_810	SA231A2	Biolegend	367733
TCR $\gamma\delta$	BUV615	11F2	BD_Biosciences	751308
Tigit	BV480	741182	BD_Biosciences	747843
Viability	LIVE/DEAD_Blue	NA	Thermo_Fisher	L34962
CD138	PerCP-Cy5.5	DL-101	BioLegend	352309
CD163	PE-Cy7	G025H7	BioLegend	353720
CD64	BV711	vi ma36	BioLegend	305041
CD80	APC	w17149d	BioLegend	375403
IgD	BV605	11-26c.2a	BioLegend	405727
IgM	BV785	mhm-88	BioLegend	314543

Supplementary Table 2.

Antibodies used to identify human immune populations via spectral flow cytometry. Surface marker panel is shown.

Supplementary Fig. S1



[illegible]

STUDY PROTOCOL

Impact of Urolithin A supplementation on mitochondrial health of immune cells (*MitoImmune*): a randomized trial

Sponsor	Amazentis SA EPFL Innovation Park, Bâtiment C 1024 Ecublens Switzerland
Sponsor Contact	Anurag Singh MD, PhD
Sponsor Study-No./ Order-No.	22.03.AMZ
Protocol Version	1.2 (17 th December, 2022)
Study Site	Universitätsklinikum Frankfurt Medizinische Klinik I, Gastroenterologie/Hepatologie; Frankfurt, Germany and Georg-Speyer-Haus – Institut für Tumorbologie und experimentelle Therapie
Study Design	A randomized, double-blind, placebo-controlled study in adults without uncontrolled chronic medical condition
Principal Investigator	Dr. med. Dominic Denk
Sub-Investigator	Dr. med. Kathrin Sprinzl, Prof. Dr.med. Stefan Zeuzem, Prof. Dr. Florian Greten
Approving IRB	The study will be reviewed by an independent institutional review board (IRB) for ethical approval. (Ethikkommission des Fachbereichs Medizin der Goethe-Universität Frankfurt am Main; Frankfurt, Germany)
Test Date(s)	June to December 2022

Background : The regulation of mitochondria is a key component of health and disease, as mitochondrial dysfunction has been associated with a host of metabolic disorders. As a result, there is a great deal of interest in interventions that can promote the creation of new mitochondria, a process known as mitochondrial biogenesis, or improve the regulation of mitochondrial quality control through mitochondrial fission, fusion and/or autophagy (mitophagy) in order to improve human health. Amazentis SA, has discovered a new natural compound called Urolithin A (UA). UA is produced by the gut microbiome following the digestion of dietary precursors (polyphenols such as ellagitannins and punicalagins) present in fruits and nuts such as pomegranate, nuts and berries. This process occurs naturally in 30-40% of the adult population that has the necessary microbiome to metabolize the dietary precursors leading to generation of UA (*European Journal of Clinical Nutrition* vol.76, pgs. 297–308 (2022)). UA has been found to impact mitochondrial health via a process called mitophagy (*Nature Medicine*, volume 22, pages 879–888 (2016)) and improve cellular and muscle health during the aging process. Amazentis has performed extensive safety

and toxicology studies with UA (*Food Chem Toxicol.* 2017 Oct; 108 (Pt A): 289-297), and also conducted a Phase 1 clinical trial that was a single ascending and multiple ascending 4-week study in healthy elderly subjects where it showed an excellent safety and bioavailability profile and modulated biomarkers of mitochondrial function in the skeletal muscle of elderly persons (*Nature Metabolism*, volume 1, pages 595–603 (2019). In a recently completed long-term (2-and 4-months) interventional clinical study (*JAMA Network Open*, 2022;5(1):e2144279), UA was found to statistically improve leg muscle endurance and improve mitochondrial and cellular health in older adults. Recent evidence around UA suggests that apart from its potent effect on mitochondrial health via activation of mitophagy, that it can also have a potent anti-inflammatory effect (enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway. *Nat Commun.* 10, 89 (2019); An increased autophagic flux contributes to the anti-inflammatory potential of urolithin A in macrophages; *Biochim Biophys Acta.* 2018 Jan;1862(1):61-70).

The emerging field of immune-metabolism has also highlighted how compromising mitochondrial function in certain immune cell populations can have detrimental consequences at the systemic level. T-cells with dysfunctional mitochondria were shown to induce premature aging (*Science* 19 Jun 2020: Vol. 368, Issue 6497, pp. 1371-1376), whereas a loss of ATP production via oxidative phosphorylation in memory T-cells exposed to chronic antigenic stimulation led to exhaustion and poor immune responses *Nat Immunol.*, 2020 Jul 13. Regulatory T-cells (Tregs) with aberrant mitochondria, led to lethal autoimmunity phenotype in rodents (*Cell Metab*, 2019 Feb 5;29(2):243-245), whereas it was shown that the anti-inflammatory cytokine, IL-10 acts via the process of mitophagy in macrophage cells to regulate immune responses (*Science*; 2017 May 5;356(6337):513-519). The purpose of this study is, therefore, to investigate the effects of Mitopure™, a commercially available source of UA, on metabolic health of immune cells and immune function in healthy adults. Specifically, we will investigate the effects of short-term (7 days) and chronic (~28 days) supplementation with UA on mitochondrial health of different immune cells populations.

Objective

To show that a natural mitophagy activator (Urolithin A) given orally can modulate mitochondrial activity in immune cells in healthy adults and this results in better immune function

Efficacy Assessment

Primary Outcome

Primary endpoints will be:

- Change in percentages of CD3+ immune cell populations. In particular, number of CD8+ T memory stem cells (identified by expression of CD8+CD45RA+CCR7+CD95+) and

naïve-like T cells (CD8+CD45RA+CCR7+CD95-)

- change in Mitochondrial activity in select immune cell populations such as CD3+ T-cells (OXPHOS activity via ELISA /Seahorse)

Secondary Outcome(s)

- Change in pro and anti-inflammatory cytokine levels (IL-6, TNF- α , IL1-B, IL-10) in plasma and/or ex-vivo antigenic stimulation
- Change in percentages of other immune cell populations (B cells, NK cells, Macrophages, DCs etc.) via flow cytometry
- Immune function assessment (Macrophage phagocytic activity, MLR, antigenic recall, etc.)
- Mitochondrial content assessment by flow cytometry in different immune-cell populations
- Transcriptomic/ proteomic/single cell analysis of selected immune cells population's (RNA-seq/qPCR for selected autophagy/mitophagy genes)
- NAD⁺ assessment in whole blood samples
- Metabolomic (acylcarnitine) analysis in plasma between the two treatment groups
- Epigenetic age of key immune cell populations/PBMCs (immune age) and biological age assessment in saliva
- Lipid profile
- Safety- adverse events recording, vital signs, biochemical and hematological profile

Type of Product(s)

- Placebo softgels
- Urolithin A softgels (1000 mg)

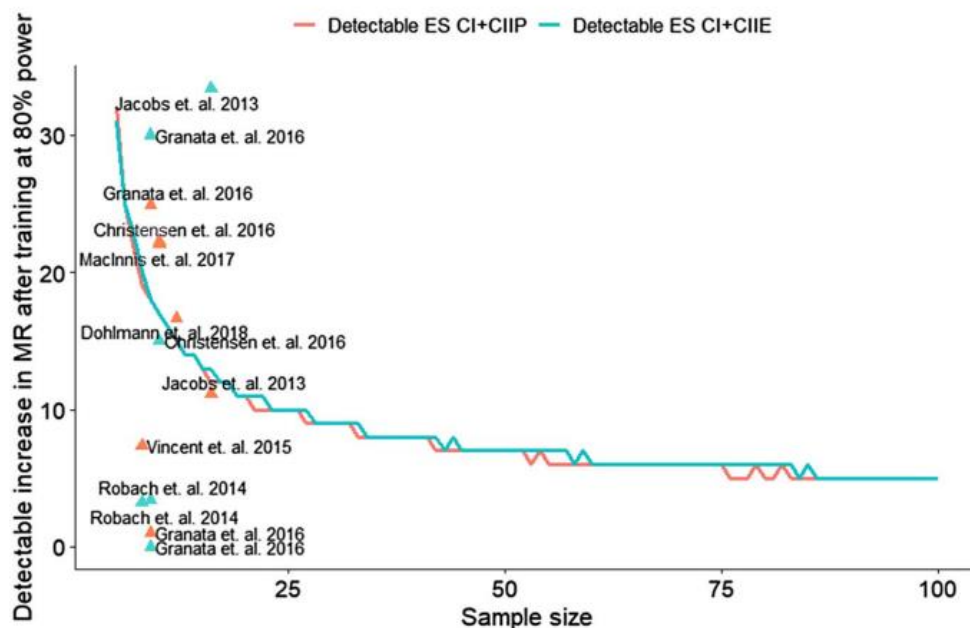
Study Design

- Randomized
- Double blind for Placebo and products
- Placebo-controlled

Sample size justification

This trial is powered to detect a difference in mitochondrial activity (i.e. OXPHOS activity) of immune cells between treatment groups. Sample size estimates were based on statistical simulations by Jacques et al 2019 (The FASEB Journal. 2020;34:2978–2986) where a comprehensive literature review was performed to assess range of effect size for 80% power when oxidative function of muscle cells was studied.

All studies used changes in mitochondrial function assessed via respirometry (i.e. mitochondrial respiration-MR) looking at either oxidation phosphorylation capacity (P) or electron transport system capacity (E). A majority of clinical studies have used sample sizes of less than n=20 participants (and are exercise training studies) and have fallen short of showing significant impact with interventions on this parameter.



An intervention that increases mitochondrial respiration by 10% at the group level based on the above simulation requires a minimum of 23 participants to detect changes for CI + CIIP and CI + CIIE at 80% power. Accounting for 10% drop-outs, we believe that n=25 subjects/arm will be sufficient to show an impact on immune cells mitochondrial respiration parameters in this study.

Likewise, the sample sizes chosen (50) is comparable to other similar studies (J Clin Invest. 2020 Nov 2;130(11):6054-6063) performed on immune health and mitochondrial function in the literature.

Type of Statistics

- Comparison to placebo-control and change from baseline in immune and mitochondrial assays of immune cell assessments.

Assessment Times

- Morning fasting intake of soft-gels prior to breakfast. Assessments to be done at baseline (V1), 7-days (V2) and after 4-weeks (V3) after intervention

Study Population

This will be a single-center, randomized, double-blind, placebo-controlled study

Total anticipated number of subjects based on sample size estimates (to be performed*):

- 50 (25/group)

Inclusion Criteria

- Healthy: Adults that do not suffer from an uncontrolled chronic medical condition that carries metabolic consequences (as assessed by the study physician)
- BMI<35kg/m²
- Provide informed consent
- Adults aged 45-70 years, both genders
- Subjects who have not received any systemic immunosuppression in the past 6 months
- Subjects with any medical condition that in the opinion of the investigators would compromise the study outcome or the safety of the research participant

Exclusion Criteria

- Subject has any concurrent medical, orthopedic, or psychiatric condition that, in the opinion of the Investigator, would compromise his/her ability to comply with the study requirements;
- Clinically significant abnormal laboratory results at screening
- Participation in a clinical research trial within 30 days prior to randomization
- Allergy or sensitivity to study ingredients
- Individuals who are cognitively impaired and/or who are unable to give informed consent
- Any condition which in the Investigator's opinion may adversely affect the subject's ability to complete the study or its measures or which may pose significant risk to the subject
- Current gastrointestinal condition which could interfere with the study (e.g. IBS/IBD, diarrhea, acid reflux disease, dysphagia etc.);
- Excessive alcohol consumption and/or a smoker
- Concomitant use of statins
- Engage in regular moderate or vigorous physically activities (i.e. Category 3 as per the IPAQ activity classification)
- Concomitant use of corticosteroids, antibiotics, any anabolic steroid, creatine, whey

protein supplements, casein or branched-chain amino acids (BCAAs), immune-boosting (Vitamin C, Zinc) or mitochondrial (COQ10, NAD+) supplements within 45 days prior to screening.

Informed Consent

For studies with subjects the following procedure will be effective: Each subject must provide the Investigator/Investigator's designee with written informed consent prior to enrollment in the study.

The original signed copy for each subject participating in the study will be retained in the Investigator's study records. The consent statement shall meet the requirements of any applicable regulation. The Investigator or the Investigator's designee will inform each subject as to the purpose and nature of the study in compliance with applicable regulations.

The subjects are informed that they can withdraw their consent at any time and that they can stop their participation in this study at any time, without disadvantages. They are also informed that proderm can also prematurely terminate their participation in the study, for example for administrative reasons.

In the case of underage subjects, the above-mentioned regulations are valid for the parents/ legal guardians of the subjects as their legal representatives in the study.

Clinical Study Schedule of Events

	Visit 1a Screening (Day-21- till Baseline)	Visit 1b Baseline Day 0	Visit 2 1 weeks of Intervention Day 7	Visit 3 4 weeks of Intervention Day 28
Informed consent	X			
Review inclusion/exclusion criteria	X			
Review medical history	X			
Height*, weight, heart rate and blood pressure, BMI	X			
Physical examination	X			
Laboratory test: CBC, HbA1c, Lipid Profile, creatinine, AST, ALT, bilirubin	X		X	X
Flow Cytometry Assessment of Immune Cell Populations		X	X	X
Immune Function (Macrophage phagocytic, MLR etc.)		X	X	X
Mitochondrial Function (OXPHOS activity on immune cells)		X	X	X
Whole Blood NAD+ assessment		X	X	X
Epigenetic age assessment (PBMCs, Saliva)		X	X	X
Intervention: Placebo/UA		X	X	X
Blood Sample (plasma- Safety, cytokines, metabolomics)		X	X	X
IP administered		X	X	X
Adverse events		X	X	X

STUDY VISITS AND ASSESSMENTS

Visit 1a: Screening (Day -30 to Day 0)

At screening, an informed consent form will be given to the potential participant. They will be required to read the information and be given the opportunity to seek more information if needed, or provided with the option of taking the consent form home to review prior to making their decision. Once consent has been obtained, the screening visit will proceed. Each participant will be sequentially assigned a screening number

Screening assessments include:

1. Informed consent signed.
2. Review of medical history, concomitant therapies and iron status.
3. Assessment of inclusion and exclusion criteria
4. Physical examination, including vital signs
5. Blood sample collection and analysis of clinical blood sample as depicted below ("study procedures")

Visit 1b: Baseline and Randomization

After confirmation that inclusion and exclusion criteria are met, participants will be randomized into UA or placebo group in a double-blinded manner. The investigative product is handed to the participants with intake instructions.

Subsequently, Visit 1b assessments include:

- Collection of questionnaire and optional stool sample
- Blood sample collection and analysis of scientific blood samples as depicted below ("study procedures")

Visit 2: After 7-days of study intervention

Visit 2 assessments include:

- Confirmation of regular intake of investigational product
- Adverse event recording
- Blood sample collection and analysis of scientific blood samples as depicted below ("study procedures")

Visit 3: After 28-days of study intervention

Visit 3 constitutes the final patient visit. Assessments include:

1. Confirmation of regular intake of investigational product
1. Adverse event recording
2. Collection of questionnaire and optional stool sample

3. Blood sample collection and analysis of both clinical and scientific blood samples as depicted below (“study procedures”)

Study procedures:

Blood sample collection: A trained physician’s assistant, nurse or physician will collect blood from the participant. A total of 30-40 ml of blood will be collected at each visit. 30ml will be used for assessment of primary and secondary endpoints (to be acquired at base, week 1 and week 4 visit; in the following identified as “scientific blood samples”), whereas baseline and week 4 visit also include additional 10ml for biochemical and hematological studies (i.e. “clinical samples”).

- 1) **Clinical blood samples** will be used for:

a) Analyses of CBC, lipid profile, creatinine, AST, ALT, bilirubin; to be performed at screening/base, as well as final visit at week 4.

- 2) **Scientific blood samples may** be used for:

a) Isolation of PBMC, separation of plasma: Blood will be layered on a Ficoll-Paque density gradient, enabling the isolation of peripheral blood mononuclear cells (PBMCs). PBMCs and plasma will be aliquoted, cryopreserved and stored at the PIs research facility for later analysis.

b) Acquisition of primary and secondary end points. Samples (PBMCs and plasma) will be used for the following experiments:

- i. **Immunophenotyping:**

-PBMCs will be stained with combinations of fluorochrome-labeled antibodies and analyzed by flow cytometry.

-4-6 randomly selected cell lysates from PBMCs will be analyzed via RNA sequencing to identify upregulated pathways governing cell fate decisions (i.e. stemness) upon Urolithin A consumption

-DNA will be extracted by PBMC to perform: quantification of mitochondrial over nuclear DNA content (mtDNA/nuDNA) and analysis of DNA methylation patterns to measure the Epigenetic/biological age

- ii Immune function assessment: PBMCs will be subjected to mixed-leukocyte reaction

(MLR) and phagocytotic activity assay for assessment of immune function upon antigen encounter.

- iii. CD3+ T cell isolations: CD3+T cells will be isolated by negative selection using antibody-labeled magnetic beads. Purified T cells will be analyzed for
 - Mitochondrial content analysis: CD3+ cells will be analyzed for mitochondrial content via Mitotracker staining using flow cytometry and/or immunofluorescence.
 - Bioenergetics analysis: CD3+T cells will be bioenergetically phenotyped using the Seahorse Extracellular Flux (XF) Analyzer and/or ELISA quantifying OXPHOS activity.
 - Characterization of metabolic control pathways: Cell lysates from CD3+ cells will be analyzed for active metabolic pathways using qPCR and Western blotting.
- iv. Plasma analysis of circulating biomarkers linked to improved mitochondrial and cellular function

Unless otherwise indicated, experiments are performed on all samples for all indicated timepoints if sample material is sufficient. Additional experiments addressing mitochondrial function, mitochondrial damage, and commitment to cell fate decision in other purified immune cell subsets may be performed at the discretion of the PI, depending on acquired results and sample volume availability.

iv. Quantification of plasma pro-inflammatory and anti-inflammatory cytokines and adipokines: We will use multiplex immunoassays to quantify levels of cytokines and adipokines.

- c) Leftover blood samples collected during this study will be stored in the PI's laboratory and used for repetition of key experiments or further analysis of subpopulations of interest.

3) Stool collection (optional): Stool collection may occur at the study location, or the sample may be collected at home and brought back to study staff for baseline and week 4 assessments. Samples collected during the study visit will be placed on ice, in an ice bath, or in a freezer until they can be delivered to the research site laboratory. Samples will be stored at -80°C. This sample may be used for quantification of fat excretion and inflammation-associated biomarkers, microbiome profiling upon UA consumption, or in future research studies involving the effect of mitophagy. This sample is optional and is not required for the completion of the study visit.

The participant will be compensated for full completion of the study. A total amount of 100€ will be provided after completion of the procedures at the study site after the week 4 visit (i.e. clinical visit,

return of questionnaire, and blood collection). Drinks may be provided during study visits following the blood draw.

INVESTIGATIONAL PRODUCT

Description of the Investigational product

Name of the product: Mitopure/Urolithin A

Form: Softgel containing 250 mg of Urolithin A along with excipients

Dose per intake: 1000 mg (4-softgels)

Timing for intake: Single oral dose administration to be orally administered daily according to the randomization for 28 days. The administration should take place in the morning, with 200 ml of tap water, in sitting position.

Product formulation:

FILL		
INGREDIENTS	AMOUNT (MG) /CAP	% TOTAL
Urolithin A	250	22.73%
Lecithin NF (35% Total PC) (Epikuron 135 F IP) - E322	284.25	25.84%
Medium Chain Triglycerides (MCT)	284.25	25.84%
Glycerol Monostearate (40-55) EP, Mono- and Diglycerides NF	11.5	1.06%
Fill weight	830 mg	75.47%
SHELL		
INGREDIENTS	AMOUNT (MG) /CAP	% TOTAL
Gelatin EP, NF	165.97	15.09%
Glycerol – E422	80.01	7.27%
Water	21.62	1.96%
Titanium Dioxide EP – E171	1.96	0.18%
DualDustmaster FD&C Blue #1 (Brilliant Blue FCF - E133)	0.234	0.021%
Sodium Copper Chlorophyllin Powder (min 95%) - E141	0.196	0.018%
Shell weight	270 mg	24.539%

Total Capsule weight	1100 mg	100%
-----------------------------	----------------	-------------

Name of the compound: Placebo

Form: Softgel containing only excipients.

Dose per intake: 4 softgel pills for blinding

Timing for intake: Single oral dose administration according to the randomization for 28 days. The administration should take place in the morning with 200 ml of tap water, in sitting position.

Product formulation:

FILL		
INGREDIENTS	AMOUNT (MG) /CAP	% TOTAL
Lecithin NF (35% Total PC) (Epikuron 135 F IP) – E322	371.49	36.14%
Medium Chain Triglycerides (MCT)	371.49	36.14%
Glycerol Monostearate (40-55) EP, Mono- and Diglycerides NF	15.03	1.46%
Fill weight	758 mg	73.74%
SHELL		
INGREDIENTS	AMOUNT (MG) /CAP	% TOTAL
Gelatin EP, NF	165.97	16.15%
Glycerol – E422	80.01	7.78%
Water	21.62	2.10%
Titanium Dioxide EP – E171	1.96	0.19%
DualDustmaster FD&C Blue #1 (Brilliant Blue FCF - E133)	0.234	0.023%
Sodium Copper Chlorophyllin Powder (min 95%) E141	0.196	0.019%
Shell weight	270 mg	26.26%
Total Capsule weight	1028 mg	100%

Unit form, packaging and labelling

This trial involves a comparison of an oral dose of Mitopure supplement versus a placebo alone for 4 weeks. The Mitopure will be provided as a maximum of 4 softgels, with each softgel containing 250mg Urolithin A or placebo.

Mitopure or Placebo softgels will be packaged into bottles, each bottle containing 30 softgels

For the purpose of the study, the daily dose of Mitopure/urolithin A or placebo the participants will be expected to take on each day 4 softgels:

- For Placebo daily dose: 4 Placebo softgels
- For 1000mg Mitopure/Urolithin A daily dose: 4 Mitopure softgels

The study labels will be inserted on side of the bottle packaging with the necessary instructions on intake along with the blinded study codes.

These will be sent to the investigational site. Each subject will receive 4 bottles for 28-day supply.

Labelling will be in accordance with local regulatory specifications and requirements.

The following information will be reported on boxes, cases and vials, in German language:

- Name, address and telephone number of sponsor,
- Name of the investigator,
- Trial reference code (Sponsor),
- Packaging batch number ,
- Supplement dosage form, route of administration, quantity of dosage units, and name/identifier and strength/potency,
- Directions for use ,
- The storage conditions,
- The mention “ Product for clinical use only” and “Do not leave within the reach of the children”.
- Blinded Study Code

The Investigator or his/her designee is responsible for the following:

- Explaining the correct use of the investigational agent(s) to the site personnel
- Verifying that instructions are followed properly
- Maintaining accurate records of investigational product dispensing and collection

Subjects will be instructed to contact the Investigator as soon as possible if he or she has a complaint or problem with the investigational product so that the situation can be assessed.

Rationale for Selection of Doses in the Study

The 1000 mg·day⁻¹ amount of UA proposed for this study has been taken in previous clinical studies (Andreux et al., Nature Metabolism, 2019) conducted by the sponsor without notable side-effects and an impact on mitochondrial health. The other ingredients in the softgels containing Urolithin A are excipients, i.e., non-primary ingredient materials, such as dietary oil for diluting or gelatin for softgels. Therefore, the Urolithin A exposure is consistent with what has been shown to be safe in previous human subject studies. As a result, the dosing regimen being employed should be well-tolerated.

Adverse Reactions

According to ICH GCP, adverse reactions (**ARs**) are defined as "all noxious and unintended responses to a medicinal product related to any dose". For clinical studies with cosmetics, consumer products or medical devices the following interpretation is adopted: The phrase "responses to a medicinal product" means that a causal relationship between the test material and an adverse event (**AE**) is at least a reasonable possibility, i.e. the relationship cannot be ruled out. Therefore, an AE which the **physician** classifies as having a causal relationship to the test material of at least 'possible' (i.e. possible, probable) is defined as an AR.

All adverse reactions (excluding those parameters being scored as part of the protocol) will be documented in the study records. The obligation to document ARs starts with enrolment of the subject in a study until 5 days following last administration of test product (if reported by the subjects).

In case of ARs it is the responsibility of the investigator to inform the Sponsor within 3 working days the latest. ARs will also be reported in the final report.

Data Management & Analysis of Data

For this study the following analysis will be defined:

DATA MANAGEMENT

All participant data will be reported in the electronic Case Report Form (eCRF), must be supported by appropriately signed identified source documentation.

Analysis Population

The investigator will decide which protocol deviations occurring in the study are assumed to be minor and major before data is analyzed. Valid subjects will be defined as enrolled subjects who will have finished the study without major deviations from the protocol and who will not have withdrawn their consent.

Sample size calculation was based on to detect changes in mitochondrial activity at 80% power, assuming a 10% dropout rate (see: sample size justification).

Missing Data

No replacement of missing data will be performed and the affected assessments will be regarded as lost for analysis.

Demographic Data

Demographic variables (age, gender) will be given for the analysis population.

Unblinding and Derandomization

No unblinding is necessary for analysis of data, because analysis will be done using the treatment codes.

Derandomization will be done by merging data to the randomization list using a unique identifier variable (e.g. randomization number, proderm identification number).

Data Listing

Raw Data

All raw data will be listed by parameter, treatment, and assessment for valid subjects only.

Calculated Values

Instrumental measurements:

- Differences to baseline will be calculated and listed by treatment and assessment time.

Descriptive Data Analysis

Summary Tables

Instrumental measurements:

- Descriptive statistics (n, mean, standard deviation, median, minimum, maximum, and 95 % confidence limits) will be given for raw data and calculated values for each treatment and assessment time.

Visual evaluation:

- Descriptive statistics (n, mean, standard deviation, median, minimum, and maximum) will be given for raw data for each parameter, treatment, and assessment time.
- Counts and percentages of single scores and scores higher than 0 will be presented in tables for raw data.

Figures

Instrumental measurements:

- The mean values of raw data and calculated values will be presented in bar charts with 95 % confidence limits for each treatment and assessment time.

Statistical Data Analysis

The analysis of primary study parameters is done using an analysis of co-variance (ANCOVA) using the factor treatment as independent factor, the baseline measurement as covariable and the change from baseline as dependent variable. 95% confidence intervals for treatment differences and corresponding non-adjusted p-values will be calculated.

A 5% significance level will be applied for the comparison of Mitopure versus Placebo.

The computation of the statistical data will be carried out with a commercially available statistics software (SAS for Windows) by an independent statistician. The study biostatistician will summarize the study data into TLFs (Table, figures and legends) primarily showing subject demographic characteristics (age, sex, ethnicity, etc.) and baseline characteristics (weight, height, concomitant medications, medical history, etc.) that will be summarized descriptively for each arm and for all subjects. The disposition of subjects includes the number and percentage of subjects in the following categories and will be summarized for each arm: subjects screened, subjects of screen failures (and the reasons for screen failures), subjects enrolled, subjects completing the trial, subjects discontinued (and the reasons for discontinuation). Continuous variables will be summarized using descriptive statistics including number of non-missing observations, arithmetic mean, standard deviation (SD), median, 25th and 75th quartile, minimum, and maximum values if not otherwise specified. Categorical variables will be summarized with frequency counts and percentages. If not specified, two-sided test will be adopted for all statistical tests and 95% confidence level will be used for all confidence intervals. The statistical analysis plan (SAP) will describe the specific details of the statistical analyses for the data collected for this study. The SAP will be finalized prior to the study unblinding. The analysis of primary efficacy parameter will be done using regression analysis using treatment arm, the baseline measurement as covariate and the change from baseline as the dependent variable. 95% confidence intervals for treatment differences and p-value will be provided. Additionally the value at each visit and change from baseline analysis for each of the primary and secondary efficacy endpoints will be summarized using descriptive statistics for each arm.

Storage of Data

All raw data pertaining to the study will be available for inspection by the Sponsor for compliance monitoring. In addition, specified scientists designated by the Sponsor may, upon appointment, examine any set of data.

All documents related to the study will be stored for at least 10 years and will be either destroyed or returned to the Sponsor afterwards, upon request.

Deviations/ Protocol Modifications

Any deviation from this protocol will be discussed with the Sponsor and appropriately documented. The investigator is responsible for any deviation from the protocol and documentation of this deviation in the study records and the final report.

If it becomes necessary to make a change to the approved protocol (i.e. signed), the investigator and the Sponsor will agree to the change before it is implemented. The change and the reason for it will be documented in an amendment. If a change is necessary on an emergency basis, the Sponsor will be notified as soon as possible after the action has been taken.

Protocol Approved:

Date:

Date:

.....
Dr. Anurag Singh (Sponsor Chief Medical Officer)

.....
Dr. Dominic Denk (PI)

The study will be carried out in conformity with legal conditions and German Human Ethics Research regulations, and with respect to GCP (ICH E6).

APPENDIX1

Declaration OF Helsinki



WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects

Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964
and amended by the:

29th WMA General Assembly, Tokyo, Japan, October 1975

35th WMA General Assembly, Venice, Italy, October 1983

41st WMA General Assembly, Hong Kong, September 1989

48th WMA General Assembly, Somerset West, Republic of South Africa, October 1996

52nd WMA General Assembly, Edinburgh, Scotland, October 2000

53rd WMA General Assembly, Washington DC, USA, October 2002 (Note of
Clarification added)

55th WMA General Assembly, Tokyo, Japan, October 2004 (Note of Clarification added)

59th WMA General Assembly, Seoul, Republic of Korea, October 2008

64th WMA General Assembly, Fortaleza, Brazil, October 2013

Preamble

1. The World Medical Association (WMA) has developed the Declaration of Helsinki as a statement of ethical principles for medical research involving human subjects, including research on identifiable human material and data.

The Declaration is intended to be read as a whole and each of its constituent paragraphs should be applied with consideration of all other relevant paragraphs.

2. Consistent with the mandate of the WMA, the Declaration is addressed primarily to physicians. The WMA encourages others who are involved in medical research involving human subjects to adopt these principles.

General Principles

3. The Declaration of Geneva of the WMA binds the physician with the words,

1/8

“The health of my patient will be my first consideration,” and the International Code of Medical Ethics declares that, “A physician shall act in the patient's best interest when providing medical care.”

4. It is the duty of the physician to promote and safeguard the health, well-being and rights of patients, including those who are involved in medical research. The physician's knowledge and conscience are dedicated to the fulfilment of this duty.

5. Medical progress is based on research that ultimately must include studies involving human subjects.

6. The primary purpose of medical research involving human subjects is to understand the causes, development and effects of diseases and improve preventive, diagnostic and therapeutic interventions (methods, procedures and treatments). Even the best proven interventions must be evaluated continually through research for their safety, effectiveness, efficiency, accessibility and quality.

7. Medical research is subject to ethical standards that promote and ensure respect for all human subjects and protect their health and rights.

8. While the primary purpose of medical research is to generate new knowledge, this goal can never take precedence over the rights and interests of individual research subjects.

9. It is the duty of physicians who are involved in medical research to protect the life, health, dignity, integrity, right to self-determination, privacy, and confidentiality of personal information of research subjects. The responsibility for the protection of research subjects must always rest with the physician or other health care professionals and never with the research subjects, even though they have given consent.

10. Physicians must consider the ethical, legal and regulatory norms and standards for research involving human subjects in their own countries as well as applicable international norms and standards. No national or international ethical, legal or regulatory requirement should reduce or eliminate any of the protections for research subjects set forth in this Declaration.

11. Medical research should be conducted in a manner that minimises possible harm to the environment.

12. Medical research involving human subjects must be conducted only by

individuals with the appropriate ethics and scientific education, training and qualifications. Research on patients or healthy volunteers requires the supervision of a competent and appropriately qualified physician or other health care professional.

13. Groups that are underrepresented in medical research should be provided appropriate access to participation in research.

14. Physicians who combine medical research with medical care should involve their patients in research only to the extent that this is justified by its potential preventive, diagnostic or therapeutic value and if the physician has good reason to believe that participation in the research study will not adversely affect the health of the patients who serve as research subjects.

15. Appropriate compensation and treatment for subjects who are harmed as a result of participating in research must be ensured.

Risks, Burdens and Benefits

16. In medical practice and in medical research, most interventions involve risks and burdens.

Medical research involving human subjects may only be conducted if the importance of the objective outweighs the risks and burdens to the research subjects.

17. All medical research involving human subjects must be preceded by careful assessment of predictable risks and burdens to the individuals and groups involved in the research in comparison with foreseeable benefits to them and to other individuals or groups affected by the condition under investigation.

Measures to minimise the risks must be implemented. The risks must be continuously monitored, assessed and documented by the researcher.

18. Physicians may not be involved in a research study involving human subjects unless they are confident that the risks have been adequately assessed and can be satisfactorily managed.

When the risks are found to outweigh the potential benefits or when there is conclusive proof of definitive outcomes, physicians must assess whether to continue, modify or immediately stop the study.

Vulnerable Groups and Individuals

19. Some groups and individuals are particularly vulnerable and may have an increased likelihood of being wronged or of incurring additional harm.

All vulnerable groups and individuals should receive specifically considered protection.

20. Medical research with a vulnerable group is only justified if the research is responsive to the health needs or priorities of this group and the research cannot be carried out in a non-vulnerable group. In addition, this group should stand to benefit from the knowledge, practices or interventions that result from the research.

Scientific Requirements and Research Protocols

21. Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature, other relevant sources of information, and adequate laboratory and, as appropriate, animal experimentation. The welfare of animals used for research must be respected.

22. The design and performance of each research study involving human subjects must be clearly described and justified in a research protocol.

The protocol should contain a statement of the ethical considerations involved and should indicate how the principles in this Declaration have been addressed. The protocol should include information regarding funding, sponsors, institutional affiliations, potential conflicts of interest, incentives for subjects and information regarding provisions for treating and/or compensating subjects who are harmed as a consequence of participation in the research study.

In clinical trials, the protocol must also describe appropriate arrangements for post-trial provisions.

Research Ethics Committees

23. The research protocol must be submitted for consideration, comment, guidance and approval to the concerned research ethics committee before the study begins. This committee must be transparent in its functioning, must be independent of the researcher, the sponsor and any other undue influence and must be duly qualified. It must take into consideration the laws and regulations of the country or countries in which the research is to be performed as well as applicable international norms and

standards but these must not be allowed to reduce or eliminate any of the protections for research subjects set forth in this Declaration.

The committee must have the right to monitor ongoing studies. The researcher must provide monitoring information to the committee, especially information about any serious adverse events. No amendment to the protocol may be made without consideration and approval by the committee. After the end of the study, the researchers must submit a final report to the committee containing a summary of the study's findings and conclusions.

Privacy and Confidentiality

24. Every precaution must be taken to protect the privacy of research subjects and the confidentiality of their personal information.

Informed Consent

25. Participation by individuals capable of giving informed consent as subjects in medical research must be voluntary. Although it may be appropriate to consult family members or community leaders, no individual capable of giving informed consent may be enrolled in a research study unless he or she freely agrees.

26. In medical research involving human subjects capable of giving informed consent, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail, post-study provisions and any other relevant aspects of the study. The potential subject must be informed of the right to refuse to participate in the study or to withdraw consent to participate at any time without reprisal. Special attention should be given to the specific information needs of individual potential subjects as well as to the methods used to deliver the information.

After ensuring that the potential subject has understood the information, the physician or another appropriately qualified individual must then seek the potential subject's freely-given informed consent, preferably in writing. If the consent cannot be expressed in writing, the non-written consent must be formally documented and witnessed.

All medical research subjects should be given the option of being informed about the general outcome and results of the study.

27. When seeking informed consent for participation in a research study the physician must be particularly cautious if the potential subject is in a dependent relationship with the physician or may consent under duress. In such situations the informed consent must be sought by an appropriately qualified individual who is completely independent of this relationship.

28. For a potential research subject who is incapable of giving informed consent, the physician must seek informed consent from the legally authorised representative. These individuals must not be included in a research study that has no likelihood of benefit for them unless it is intended to promote the health of the group represented by the potential subject, the research cannot instead be performed with persons capable of providing informed consent, and the research entails only minimal risk and minimal burden.

29. When a potential research subject who is deemed incapable of giving informed consent is able to give assent to decisions about participation in research, the physician must seek that assent in addition to the consent of the legally authorised representative. The potential subject's dissent should be respected.

30. Research involving subjects who are physically or mentally incapable of giving consent, for example, unconscious patients, may be done only if the physical or mental condition that prevents giving informed consent is a necessary characteristic of the research group. In such circumstances the physician must seek informed consent from the legally authorised representative. If no such representative is available and if the research cannot be delayed, the study may proceed without informed consent provided that the specific reasons for involving subjects with a condition that renders them unable to give informed consent have been stated in the research protocol and the study has been approved by a research ethics committee. Consent to remain in the research must be obtained as soon as possible from the subject or a legally authorised representative.

31. The physician must fully inform the patient which aspects of their care are related to the research. The refusal of a patient to participate in a study or the patient's decision to withdraw from the study must never adversely affect the patient-physician relationship.

32. For medical research using identifiable human material or data, such as research on material or data contained in biobanks or similar repositories, physicians must seek informed consent for its collection, storage and/or reuse. There may be exceptional situations where consent would be impossible or impracticable to obtain

for such research. In such situations the research may be done only after consideration and approval of a research ethics committee.

Use of Placebo

33. The benefits, risks, burdens and effectiveness of a new intervention must be tested against those of the best proven intervention(s), except in the following circumstances:

Where no proven intervention exists, the use of placebo, or no intervention, is acceptable; or

Where for compelling and scientifically sound methodological reasons the use of any intervention less effective than the best proven one, the use of placebo, or no intervention is necessary to determine the efficacy or safety of an intervention

and the patients who receive any intervention less effective than the best proven one, placebo, or no intervention will not be subject to additional risks of serious or irreversible harm as a result of not receiving the best proven intervention.

Extreme care must be taken to avoid abuse of this option.

Post-Trial Provisions

34. In advance of a clinical trial, sponsors, researchers and host country governments should make provisions for post-trial access for all participants who still need an intervention identified as beneficial in the trial. This information must also be disclosed to participants during the informed consent process.

Research Registration and Publication and Dissemination of Results

35. Every research study involving human subjects must be registered in a publicly accessible database before recruitment of the first subject.

36. Researchers, authors, sponsors, editors and publishers all have ethical obligations with regard to the publication and dissemination of the results of research. Researchers have a duty to make publicly available the results of their research on human subjects and are accountable for the completeness and accuracy of their reports. All parties should adhere to accepted guidelines for ethical reporting. Negative and inconclusive as well as positive results must be published or otherwise made

7/8

publicly available. Sources of funding, institutional affiliations and conflicts of interest must be declared in the publication. Reports of research not in accordance with the principles of this Declaration should not be accepted for publication.

Unproven Interventions in Clinical Practice

37. In the treatment of an individual patient, where proven interventions do not exist or other known interventions have been ineffective, the physician, after seeking expert advice, with informed consent from the patient or a legally authorised representative, may use an unproven intervention if in the physician's judgement it offers hope of saving life, re-establishing health or alleviating suffering. This intervention should subsequently be made the object of research, designed to evaluate its safety and efficacy. In all cases, new information must be recorded and, where appropriate, made publicly available.

Disclaimer: © 2013 World Medical Association, Inc. All Rights Reserved. All intellectual property rights in the Declaration of Helsinki are vested in the World Medical Association. Previous permissions granted to publish or reproduce otherwise WMA policy don't apply to this version of the Declaration of Helsinki until January 1st, 2014. For further questions, please contact the WMA secretariat at secretariat@wma.net.

© World Medical Association, Inc. - All Rights reserved.
© Asociación médica mundial - Todos los derechos reservados.
© L'Association Médicale Mondiale - Tous droits réservés.

