Study protocol

TirolGESUND

General Exercise & Smoking Undone & Nutrition Diet

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Sponsor

Tirol Kliniken GmbH.

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Biometrics

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Date of the version: 02/09/2023

Version: 6

Signature of the principal examiner:

Signature of the sponsor:

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- LFU - Leopold-Franzens-University Innsbruck
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- MUI - Medical University of Innsbruck
- ISAG - Institute for Sports, Alpine Medicine and Health Tourism
- SHT - Addiction support Tyrol
- UMIT - Private University for Health Sciences, Medical Informatics and Technology

List of abbreviations

BMI	Body mass index
DNAme	DNA methylation
GCP	Good clinical practice
GDPR	General Data Protection Regulation
IF	Intermittent fasting
P4 Medicine	Predictive, preventive, personalised and participatory medicine
PBMC	Mononuclear cells of the peripheral blood (Peripheral blood-derived mononuclear cells)
PPC	Posterior predictive check
TirolGESUND	Tirol General Exercise & Smoking Undone & Nutritious Diet
WID index	Women's cancer risk identification Index

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Synopsis of the study

Study management	University Professor Dr Martin Widschwendter EUTOPS Institute
Study title	Tirol - General Exercise & Smoking Undone & Nutritional Diet
Abbreviation of the study title	TirolGESUND
Protocol version and date	Version 6 (dated 02/09/2023)
Hypothesis	Lifestyle interventions such as smoking cessation or intermittent fasting (with or without ketogenic supplementation) can lead to changes in the WID methylation index in cervical cells.

Endpoints	Primary endpoint:
	• Absolute difference in the WID index score of the DNA methylation signature in cervical smears before and after intervention (6 months)
	
	Secondary endpoints (exploratory):
	• Changes in DNA methylation with regard to WID index scores, age-associated signatures and genome-wide methylation in cervical smears before, during and after the intervention (0, 2, 4, 6, 12, 18, 24 and 30 months) to assess longitudinal effects and the temporal dynamics of any changes in DNA methylation
	• Changes in DNA methylation in blood, oral mucosa and menstrual blood (the latter only optional in premenopausal menstruating women) with regard to age-associated DNA methylation signatures and genome-wide methylation before, during and after the intervention (0, 2, 4, 6, 12, 18, 24 and 30 months)
	• Absolute change in DNA mutation load in cervical and oral mucosal swabs, blood, and menstrual blood (genome-wide)
	• Change in clonal haematopoiesis of indeterminate potential (CHIP), measured via mutation analysis of DNMT3A, ASXL1, JAK2 and TET2 before and after the intervention (6, 12, 18, 24 and 30 months)
	• Change in cell identities and cell composition of the blood (Single Cell Sequencing - RNA + ATAC-Seq; sub-study)
	• Change in clinical factors before and after the intervention (0 and 6, 12, 18, 24 and 30 months):
	  ○ Smoking cessation intervention: Smoking status
	  ○ Nutritional intervention: BMI, body composition
	  ○ Sports medicine parameters
	• Absolute or relative changes in various blood values over the course of the intervention (0, 2, 4, 6, 12, 18, 24 and 30 months), including
	  ○ Lipids, HbA1c, metabolic profile
	  ○ Cellular measurements: distribution of different immune cell populations (% and absolute values) in peripheral blood mononuclear cells (PBMCs); activation levels of monocytes and T cells; expression of adhesion molecules
	  ○ Inflammatory cytokines and factors and blood (plasma), including IL-1, HMGB-1, RAGE, sASC, IL-6, etc.
	  ○ Cytotoxicity of peripheral blood mononuclear cells
	• Changes in the vaginal, faecal and oral microbiome during and after completion of the intervention
	• Changes in the metabolic profile in urine, faeces and saliva during and after completion of the intervention
	• Changes in epigenetic and functional characteristics of extrinsic skin ageing and skin barrier, their reversion by lifestyle changes and a comparison of both methods of determining the biological age of the skin before and after the intervention (0 and 6 months)

	• Change in stem cell fitness of endometrial stem cells from menstrual blood with regard to pluripotency and colony formation potential, etc. (0 and 6, 12, 18, 24 and 30 months) • Change in vascular health factors (pulse wave velocity, intima-media thickness, plaque score) before and after the intervention (0 and 6, 12, 18, 24 and 30 months) • Absolute change in the abdominal fat composition (visceral and subcutaneous) • Change in EQ-5D-5L scores with regard to health status and quality of life before and after the intervention (0 and 6, 24 and 30 months) • Change in psychological scale scores before and after the intervention (0 and 6, 24 and 30 months) • Analysing psychological factors that determine compliance and effectiveness of the intervention with regard to lifestyle changes • Reprogramming of peripheral blood cells and investigation of epigenetic reprogramming signatures after the intervention
Study design	Prospective, longitudinal, non-therapeutic intervention study without control group over 6 months (optional follow-up visits 12 and 18 as well as 24 and 30 months)
Statistical analysis	Values from each individual subject will be used as a baseline (control) for longitudinal measurements. For the smoking intervention, values are compared longitudinally. For the dietary intervention, subjects will be randomised 1:1 to the two intervention arms (intermittent fasting or intermittent fasting with ketogenic supplement) and compared both longitudinally and between these two groups. Linear mixed regression models will be used for the statistical evaluation in relation to epidemiological and clinical values (BMI, markers of chronic inflammation, estimated ovulation cycles, etc.).
Inclusion and exclusion criteria	Inclusion criteria*: 1. Women between the ages of 30 and 60 2. motivates people to change their lifestyle 3. Smoking cessation: >10 cigarettes per day and for at least 5 years 4. Intermittent fasting: BMI between 25 and 35 * If inclusion criteria 3 and 4 apply, the subject is assigned to the smoking cessation group. Exclusion criteria are: 1. Relevant previous illnesses: a. Current and/or past malignant cancers b. Current and/or past cardiac diseases c. Current and/or past metabolic diseases d. Current and/or past psychiatric illnesses 2. Current pregnancy and breastfeeding period 3. Women with total hysterectomy 4. Women with a known premalignant (CIN2/CIN3) or malignant change (invasive cancer) of the cervix (currently and/or in the past) 5. Simultaneous participation in another interventional study The study is also cancelled for the individual if exclusion criteria 1b, c, d, 2, 3, and 5 occur.
Number of cases	The TirolGESUND study will examine a total of 180 women (n=60 for smoking cessation; n=60 for intermittent fasting alone; n=60 for intermittent fasting with ketogenic supplement).
Intervention	Smoking cessation: • Three appointments in a group setting (6-12 participants per session) • Two individual telephone counselling sessions with an addiction counsellor • Supportive exercise protocol and dietary advice (start, month 2 and 4) to promote a balanced diet • Regular 1:1 coaching Intermittent fasting:

	• Initiation (1 month) and maintenance (5 months) of a 16:8 intermittent fasting regime (time restricted feeding) • Dietician consultations with clinical dieticians after 2 and 4 months to analyse nutritional behaviour and motivate continuation • A further dietician consultation after 6 months to gradually stop or continue intermittent fasting • Supporting movement protocol • Regular 1:1 coaching Intermittent fasting with a ketogenic supplement: • Initiation (1 month) and maintenance (5 months) of a 16:8 intermittent fasting regime (time restricted feeding) • Dietary counselling appointments with clinical dieticians to maintain intermittent fasting with ketogenic supplementation after 2 and 4 months to analyse dietary behaviour and motivate continuation • Ketogenic supplement (MCTfiber by Kanso) • A further dietician consultation after 6 months to gradually stop or continue intermittent fasting • Supporting movement protocol • Regular 1:1 coaching
Study investigations	During the course of the study, several study investigations will be carried out. Baseline (T0): • General health and fitness check incl. medical history and exercise ergometry • Collection of biomaterials: cervical smear, blood, urine, saliva and stool samples, oral mucosa smear, skin biopsy, menstrual blood sample # • Assessment of vascular health through pulse wave velocity measurement and carotid sonography • Measurement of visceral and subcutaneous abdominal fat • Skin biopsy § • Assessment of psychological factors and health-related quality of life (EQ-5D-5L) • Nutrition check, dietary councelling & bioimpedance measurement * Month 2 (T2): • Collection of biomaterials: cervical smear, blood, urine, saliva and stool samples, oral mucosa smear, menstrual blood sample # • Nutrition check, dietary councelling & bioimpedance measurement * Month 4 (T4): • Collection of biomaterials: cervical smear, blood, urine, saliva and stool samples, oral mucosa smear, menstrual blood sample # • Nutrition check, dietary councelling & bioimpedance measurement * Final measurement (T6): • General health and fitness check incl. medical history • Collection of biomaterials: cervical smear, blood, urine, saliva and stool samples, oral mucosa smear, skin biopsy, menstrual blood sample # • Nutrition check, dietary councelling & bioimpedance measurement * • Assessment of vascular health through pulse wave velocity measurement and carotid sonography • Measurement of visceral and subcutaneous abdominal fat • Skin biopsy § • Assessment of psychological factors and health-related quality of life (EQ-5D-5L) Optional: Month 12 (T12):

	• Collection of biomaterials: cervical smear, blood, urine, saliva and stool samples, oral mucosa smear, skin biopsy§, menstrual blood sample # • Assessment of psychological factors and health-related quality of life (EQ-5D-5L) Optional: Month 18 (T18): • Collection of biomaterials: cervical smear, blood, urine, saliva and stool samples, oral mucosa smear, skin biopsy§, menstrual blood sample # • Assessment of psychological factors and health-related quality of life (EQ-5D-5L) Optional: months 24 and 30: • Collection of biomaterials: cervical smear, blood, urine, saliva and stool samples, oral mucosa smear, skin biopsy§, menstrual blood sample # • Assessment of psychological factors and health-related quality of life (EQ-5D-5L) • Recording compliance with the interventions • Survey of smoking or eating behaviour • Declaration of consent for follow-up visits, access to the tumour register and contact for presentation of results Continuous (test person at home): • β-hydroxybutyrate in capillary blood* • Fitness tracker for measuring the number of steps, basal heart rate and estimated calorie consumption # optional for premenopausal women? § optional, requires separate consent (obtained by the Department of Dermatology) * only some subjects in study arms "intermittent fasting" and "intermittent fasting + ketogenic supplement" as of version 4
Duration and schedule of the study	The study is planned over a period of 36 months. A period of 30 months is planned from the first screening visit of the first participant to the completion of the measurements for the last subject. Analyses and statistical evaluation will continue until 6 months after completion of the clinical measurements. • Q1 2021 first-participant-in • Q2 2024 last-participant-out
Study centre	European Oncology Prevention & Screening Institute Phone: +43 […] (Prof. Dr Martin Widschwendter) email: info@eutops.at or […] Leopold Franzens University Innsbruck Milser Str. 10, 6060 Hall
insurance	Zurich Insurance Company Ltd Schwarzenbergplatz 15 A-1010 Vienna Tel. no. +43 8000 80 80 80 Policy number: 07208763-1
Ethical aspects	No side effects or health risks for the participants are expected during the course of the TirolGESUND study. The study will provide potential evidence of the effectiveness of health-promoting and disease-preventing interventions and thus bring comprehensive value to the health of society. Participation in the study is also expected to have potential health benefits for the subjects themselves.

1. Scientific foundations

1.1. Paradigm shift to P4 medicine

Despite many successes in treatment and diagnosis over the last 30 years, cancer remains one of the most common causes of death in high-income countries [1]. Hormone-sensitive, female-specific cancers, including breast, ovarian, endometrial and cervical cancers, account for 42% of all cancers in women [2]. Every year, 773,000 new diagnoses of these cancers are made in the EU, and breast cancer alone represents an economic and socio-economic burden of € 15 million [3]. Although huge progress has been made in cancer screening and treatment in recent years, some of the cancers mentioned above are still only detected at a late stage (e.g. ovarian cancer) and subsequently have a 5-year survival rate of less than 40%[4]. In addition, only very modest improvements in the 5-year survival of this type of cancer have been achieved in the last 30 years (approx. 2-4%) [5,6].

Medicine is currently undergoing a transition from a reactive to a proactive discipline: instead of reacting to symptoms, the concept of "P4" medicine (predictive, preventive, personalised and participatory) seeks to promote health before diseases develop [7]. This paradigm shift is made possible by technological advances and insights from systems biology. An excellent example of a successful implementation of this concept is the cardiovascular field: mortality as a result of coronary heart disease has fallen dramatically over the past thirty years [8]. This is mainly a consequence of the fact that risk factors such as high blood pressure and cholesterol levels a) can be measured directly and by simple and non-invasive methods, b) can be modulated by pharmacological intervention (e.g. antihypertensives, statins) or lifestyle changes, and c) the effectiveness of such interventions can be monitored by simple methods (see a)). To this end, the cardiovascular community has recognised that absence of symptoms does not necessarily mean absence of disease.

Such a paradigm shift has not yet taken place in cancer medicine. Although some risk factors, such as tobacco consumption [9] or diet (directly or indirectly through obesity) [10–12] are now well established and widely known, not everyone with these risk factors will develop cancer. Equally, individuals without these risk factors may develop cancer in the future. The aetiology of cancer is a complex interplay of intrinsic genetic predisposition and external factors such as environmental, behavioural and lifestyle-associated factors, as well as random somatic mutations with increasing age [13–15]. However, genetic predisposition (depending on the type of cancer) contributes on average less to the development of cancer, and risk prediction based purely on genetic factors is therefore relatively weak. The complexity and high individual component of cancer development makes risk prediction more complex than for cardiovascular diseases. A risk stratification test for cancer should

therefore ideally a) take into account both genetic and non-genetic factors, b) be performed by simple and non-invasive procedures using easily accessible tissue samples in order to be suitable for population-based screening, and c) offer the possibility of verifying the effectiveness of risk-reducing measures [16].

1.2. Epigenetics and risk prediction

The field of epigenetics describes an additional level of genetic information to DNA, which is made possible by superimposing chemical modifications on the DNA. Epigenetic modifications (DNA methylation, histone modifications and non-coding RNAs) enable genetically identical cells to express different and stable phenotypes by allowing the epigenome to control the accessibility of different parts of the genome to the transcription machinery [17,18]. Epigenetic modifications, like genetic material, can be inherited mitotically and meiotically, but unlike genetic factors, the epigenome is cell type specific and can be modified during development and by external factors [19]. DNA methylation (DNAme) at CpG dinucleotides in the C5 position is the most stable and easily measurable type of epigenetic modification. DNAme can be modified by many external factors, for example tobacco consumption, ageing processes, nutrition, chronic inflammation or exposure to toxic substances [20–26]. In addition, aberrant DNAme, characterised by *hypermethylation* at CpG promoter regions and global *hypomethylation*, is a common occurrence in cancer cells [27–29].

As the epigenome integrates information from genetic factors and environmental influences, it has emerged as an attractive target for cancer risk prediction. Epigenetic changes can already exist years before the development of cancer [30,31] and are strongly associated with the development of cancer [32–35]. DNAme is cell type or tissue specific, which is why the "FORECEE" prevention research project for women-specific cancers (www.forecee.eu) has implemented cervical smears as surrogate tissue, as these capture hormone-sensitive epithelial tissue, which is easily accessible, can be obtained minimally invasively and is often available through routine examinations or HPV screening. Recent research results of the Widschwendter group within this "FORECEE" project describe DNA methylation signatures that could indicate the cancer risk for four female-specific cancers. These DNA methylation signatures of the cervix are called WID indices (for "Women's cancer risk IDentification" indices), including WID-BC (WID-Breast cancer; breast cancer), WID-OC (WID-Ovarian cancer; ovarian cancer), WID-EC (WID-Endometrial cancer; endometrial cancer), and WID-CIN (WID-Cervical intraepithelial neoplasia; cervical cancer) (unpublished, from the Widschwendt group; currently in the review process for scientific publication). The four WID scores are elevated in women with the respective cancers and have a high sensitivity and specificity for differentiating women with or without cancer. A population-based prospective validation of these

scores is being planned and is running in parallel to the TirolGESUND study, but there are initial indications that WID scores not only recognise current cancers, but also indicate increased risk (and their reduction could indicate a lower risk): the WID-BC index is measured in healthy women with mutations in the *BRCA1/2* genes (who have an increased risk of breast cancer). Similarly, the WID-CIN index can distinguish women who develop CIN3+ 1-4 years after sample collection from women who do not develop CIN3+ (in cytologically negative, HPV+ samples). Interestingly, unlike many other current DNA methylation tests, WID scores are not based on the presence of tumour DNA, indicating that WID scores are not (purely) screening tests, but actually indicate a systemic increased risk of cancer for the disease in question.

The team has also developed specific epigenetic indices that reflect biological ageing, smoking and BMI, for example. Age is one of the most important risk factors for cancer development, and DNA methylation plays a major role here. DNA methylation is highly correlated with chronological age: normal cells show a gradual "epigenetic drift" as they age [36,37]. Epigenetic clocks, e.g. according to Horvath [38] or Hannum [39] can estimate the chronological age based on the DNA methylation of certain genes. However, external factors can lead to a discrepancy between the "epigenetic" and chronological age, which is referred to as epigenetic age deceleration (epigenetic age < chronological age) or age acceleration (epigenetic age > chronological age). Obesity, menopause, and psychosocial factors (low socioeconomic status or psychosocial adversities in childhood) can lead to age acceleration in cells of the blood (menopause; psychosocial factors) [40,41] or the liver (obesity) [42]. In contrast, smoking leads to age deceleration in cells of the oral mucosa [43]. Biological ageing also leads to the methylation of polycomb group protein target genes (PCGTs), which are also preferentially methylated in cancer cells. Methylation in these genes results in an irreversible stabilisation of stem cell phenotypes and an increased division rate of these cells. This age-associated "PCGT signature" is found in preneoplastic conditions and can contribute to the development of cancer [44]. Based on PCGT methylation, an epigenetic mitotic clock was therefore defined, which is referred to as "epiTOC" and can indicate the division rate of cells. In cancer cells, preneoplastic cells, and normal epithelial cells exposed to tobacco carcinogens, epiTOC indicates an acceleration of epigenetic age and mitotic rate [45]. We will therefore use DNA methylation in the course of the study to assess cancer risk and biological ageing.

1.3. Modifiable risk factors and initial study results

Tobacco consumption and diet (directly or indirectly via body mass index) are established risk factors for (women-specific) cancers [9,10,12,46–57]. Tobacco contains over 70 known DNA mutagens, and studies report that smoking also alters the cellular epigenome [58]. Obesity and certain diets,

especially the "Western diet", can result in a chronic inflammatory body state with elevated levels of reactive oxygen species [10,59]. Diet or related factors are associated with 30% of all cancer cases [11] and an elevated BMI (>25) is associated with over 20% of all cancer deaths in women [60]. However, both tobacco use and diet are considered *modifiable* risk factors as they are behavioural or lifestyle risk factors.

Research in animal models has shown that a cyclical ketogenic diet or intermittent fasting reduces BMI (and thus lowers the risk of cancer), but also has other metabolic benefits: in mice, these interventions extend lifespan and simultaneously reduce the incidence of tumours [61–64]. In parallel, there is also initial evidence of a beneficial effect of intermittent fasting on general health in humans (summarised in [65]). In a prospective clinical study, intermittent fasting for only three months reduced risk biomarkers for cancer and inflammation, such as IGF-1 and CRP [63]. A cross-sectional clinical study also reported that quitting tobacco can reduce the risk of cancer [66]. Ketogenic diet therapy is already known in the treatment of (paediatric) epilepsies [67] and may also be beneficial as an adjuvant therapy for aggressive cancers such as glioblastoma multiforme [68]. Ketone bodies, which occur in ketosis, can have a metabolic effect on (stem) cells [69,70] which is why the ketogenic diet is now gaining interest for research both in combination with intermittent fasting and on its own.

Both tobacco consumption and diet can alter the epigenome and lead to clearly defined signatures (e.g. "smoking" or "obesity signature" [58]; partly unpublished, Widschwendter group), but it is not yet clear whether these risk-specific signatures can be reversed by lifestyle intervention - for example, smoking cessation and intermittent fasting +/- ketogenic supplementation. Initial data from animal models and cross-sectional studies in patients suggest that this may be the case [71,72]. However, longitudinal studies in patients in this area are still lacking. In addition, it is unknown whether and to what extent individual factors contribute to a reduction in cancer risk (i.e. not every person reacts in the same way to external factors).

In the present longitudinal study, we will investigate whether epigenetic signatures in cervical cell smears (and in other biological samples) can be modulated by lifestyle interventions - smoking cessation and intermittent fasting +/- ketogenic supplement - and thus could indirectly indicate a reduced cancer risk. We use a ketogenic supplement to facilitate the achievement of ketosis and to standardise between subjects, which would not necessarily allow a ketogenic diet without strict control of dietary protocols. If we can measure a reduction in WID index scores through this intervention, results from this study will provide the first basic evidence that DNA methylation and

mutational load can be modulated by lifestyle interventions, and potentially the effectiveness of preventive measures can be tested using relatively simple and non-invasive methods.

Both tobacco consumption [73] as well as diet [74,75] (directly and indirectly) are not only risk factors for cancer, but also for general mortality. Worldwide, one in five deaths (totalling approximately 11 million annually) is associated with suboptimal diets; high sodium intake and low consumption of fruits, whole grains and nuts are leading risk factors for suboptimal diets [74]. Indirectly influenced by diet, the risk of diseases of the cardiovascular system, digestive system, musculoskeletal system and urogenital tract increases proportionally from a BMI of >25. [76]. On average, the remaining life expectancy of smokers is reduced by 11 years compared to non-smokers [73]. Therefore, in addition to investigating the change in WID index scores with regard to female-specific cancers, this study will also serve as a pilot project for the prevention of other diseases. This pilot project will be made possible through co-operation with relevant experts in various fields. We will describe a variety of clinical, epidemiological, psychological and biochemical factors in the course of the interventions in order to generate initial exploratory data for further preventive studies.

2. Study objectives

Tobacco consumption and diet are significant risk factors for the four female-specific hormone-sensitive cancers (breast, ovarian, endometrial and cervical cancer), but there is so far only sparse longitudinal evidence for the reduction of a potentially increased cancer risk through lifestyle changes. In particular, it is not clear how much individual factors contribute to the effectiveness of such preventive measures. The TirolGESUND study aims to provide evidence that smoking cessation and intermittent fasting (+/- ketogenic supplementation) over 6 months can lead to changes in DNA methylation, particularly in the context of a reduction in disease-revealing signatures. In this regard, longitudinal WID index scores, which can indicate the cancer risk for four women-specific cancers (breast, ovarian, endometrial and cervical cancer), will be investigated. Furthermore, we are interested in whether interventions alter age-associated DNA methylation signatures (especially age acceleration/deceleration). We will also investigate how modulatable DNA mutational load is (indirectly, for example by replacing mutation-loaded cells with new, "healthy" and non-mutated stem cells). Our primary endpoint is DNA methylation changes between 0 and 6 months, however, by further sampling at 2 and 4 months we will investigate the more precise temporal resolution of potential DNA methylation changes and prevent results that could arise from "regression to the mean". The time frame of the study was determined based on the results of preliminary studies on DNA methylation plasticity, which have shown changes within 3-6 months [77–80]. After the intervention phase follow-up visits at intervals of 6, 12, 18 and 24 months were conducted in order to investigate the

adherence of the subjects to the interventions and the durability of the lifestyle change on the one hand and the sustainability of the effects of the interventions on the other.

There are three intervention groups in the TirolGESUND study: Smoking cessation, intermittent fasting, and intermittent fasting with ketogenic supplementation. After extensive discussion, the study management and partners decided against a control group ("standard of care"). Participation in TirolGESUND is a considerable effort for participants, who have to come for several examinations over a period of 30 months, including invasive sample collection (e.g. blood samples). However, the interventions are likely to result in direct benefits for participants (e.g. successful smoking cessation, BMI reduction) and participants will be actively supported by a professional team of dieticians and addiction counsellors and will receive 1:1 support from personal coaches. We do not anticipate being able to recruit a sufficient number of women who will not receive any such benefit from their participation, but who will still come back several times to provide samples. It would also be unethical, for example, to recruit a group of smokers without intervention and instruct them *not to* stop smoking for 6 months. Furthermore, potentially interested subjects in a randomised "standard of care" group would presumably still concern themselves with their health and possibly still smoke less or practice intermittent fasting, as participation in TirolGESUND presupposes a certain health and preventive interest. In this respect, the inclusion of a control group is not trivial or feasible, and we have instead opted for a longitudinal study per subject, which is made possible by longitudinal statistical analysis. Such a strategy has already been established in several previous studies on the longitudinal investigation of biological markers (without control groups) [77,78,80].

As smoking and diet are risk factors for many other diseases - for example diseases of the digestive tract (inflammatory bowel disease), diseases with a vascular component (stroke, heart attack, dementia), or skin diseases (skin cancer, psoriasisand premature skin ageing) - the study also aims to be an exploratory pilot study of clinical, epidemiological, psychological, cellular and biochemical factors associated with these diseases during the course of the interventions. These exploratory data will lead to hypotheses for further lifestyle intervention studies of the P4 concept. The study also promises to provide unique data to determine the coherence (or non-coherence) of the epigenetic markers of biological ageing with the functional ageing parameters (collagen changes, extracellular matrix changes in the dermis and epidermis, epidermal barrier function, functional interactions between dermis and epidermis, etc.) obtained directly from skin biopsies. Of particular scientific interest in this context is the question of the extent to which the reversion of ageing characteristics ("rejuvenation") through smoking cessation and/or intermittent fasting can be

reflected in the epigenetic and "functional" parameters and whether both types of ageing parameters reflect the above-mentioned reversion in the same way.

In addition, we will also be the first study to investigate the effect of lifestyle interventions on endometrial stem cells. These stem cells have similar characteristics to mesenchymal stem cells (MSCs) [81,82] but, unlike MSCs, can be harvested from menstrual blood in a minimally invasive manner. It has already been demonstrated that factors such as ageing [83,84], tobacco consumption [85], overweight or obesity [86], and diet (intermittent fasting/ketogenic diet) [87] can influence the regenerative potential of stem cells (e.g. mutational load, proliferation, differentiation). We will investigate whether stem cells obtained after the intervention are "rejuvenated" (in terms of DNA methylation profile and mutational load) or show higher regenerative capacity - first indications of an increase in potential already exist in animal studies, especially for intermittent fasting and ketogenic diet [88–90].

Ultimately - for cost reasons in a sub-study of 20 participants - we will use "single cell sequencing", i.e. analysing individual cells, to investigate the cell heterogeneity of the blood. It is becoming increasingly clear that an "ageing" or inadequate immune system contributes to the development of diseases such as cancer [91], neurodegenerative diseases [92], or - currently - severe courses of COVID-19 [93]. Using a multi-omics approach, in which both gene expression and chromatin accessibility of individual cells can be recorded simultaneously, we will investigate the heterogeneity of the blood and specific cell identities before intervention and after 2 months. This approach allows us, for example, to determine the number of ageing cells in the blood and whether a change occurs at the level of individual cells: most of our molecular analyses (DNA methylation, DNA mutation analysis) are performed in "bulk", i.e. it is not possible to determine with complete certainty whether a change in DNA methylation is due to a) a change in cell composition or b) a change in the methylation levels of individual cells (or both). Using single cell sequencing, we achieve the required fine granularity of data, which allows interesting new insights into the physiological changes of the immune system after lifestyle intervention. Furthermore, in this context - immune system and immune surveillance - we record the cytotoxicity of peripheral blood mononuclear cells.

2.1. Hypotheses and target criteria

The basic hypothesis of the TirolGESUND study is that relatively short-lasting lifestyle interventions for 6 months - smoking cessation and intermittent fasting +/- ketogenic supplementation - will induce comprehensive biological changes and lead to a reduction of WID index scores in cervical DNA. In addition to smoking cessation and intermittent fasting +/- ketogenic supplementation, we will

implement a supportive exercise programme, as physical activity also leads to a reduction in the risk of several diseases, including cancer, diabetes, stroke, and myocardial infarction [94,95].
The 6-month period (with interim visits at 2 and 4 months) is based on previous preliminary studies on DNA methylation plasticity by the Widschwendter group and other research groups, which suggest that DNA methylation changes occur within 3-6 months [78–80,96]. The subsequent follow-up visits at 6, 12, 18 and 24 months after the end of the intervention phase are intended to investigate the sustainability of the effects and to check the adherence of the participants to the interventions.

2.1.1. Primary target criterion

The primary objective of this study is to provide longitudinal evidence that lifestyle changes over time lead to a reduction in WID indices that can potentially indicate cancer risk for four female-specific cancers (WID-BC, WID-OC, WID-EC, WID-CIN). By taking multiple cervical smears over the duration of the lifestyle interventions, epigenetic WID indices are determined for each proband (and their change compared to the baseline before the intervention). The absolute reduction of the WID index for the four different female-specific cancers - breast, ovarian, endometrial and cervical cancer - and age-dependent DNA methylation before and after 6 months of intervention is considered the primary endpoint of the study.

2.1.2. Secondary target criteria

At the same time, this study will serve as an exploratory pilot project for further prospective studies on risk reduction and general health promotion. By involving different experts in this study, we will create a biological database for further hypothesis generation and measure inter-individual variations on interventions. Further studies on the promotion of different aspects of health through diet, exercise and behaviour change will follow.

We will include the following secondary outcome measures as an exploratory study for research and development:

- Changes in DNA methylation with regard to WID index scores, age-associated signatures and genome-wide methylation in cervical smears before, during and after the intervention (0, 2, 4, 6, 12, 18, 24 and 30 months) to assess longitudinal effects and the temporal dynamics of any changes in DNA methylation
- Changes in DNA methylation in blood, oral mucosa and possibly menstrual blood (the latter only optional in premenopausal menstruating women) with regard to age-associated DNA methylation signatures and genome-wide methylation before, during and after the intervention (0, 2, 4, 6, 12, 18, 24 and 30 months)

- Absolute change in DNA mutation load in cervical and oral mucosal swabs, blood, and menstrual blood (genome-wide)
- Change in clonal haematopoiesis of indeterminate potential (CHIP), measured via mutation analysis of DNMT3A, ASXL1, JAK2 and TET2 before and after the intervention (0?, 6, 12, 18, 24 and 30 months)
- Change in cell heterogeneity and number of ageing cells (using single cell sequencing; sub-study)
- Change in clinical factors before and after the intervention (0, 6, 12, 18, 24 and 30 months):
    - Smoking cessation: Smoking status
    - Nutritional intervention: BMI, body composition
    - Sports medicine parameters
- Absolute or relative changes in various blood values over the course of the intervention (0, 2, 4, 6, 12, 18, 24 and 30 months), including
    - Lipids, HbA1c, metabolic profile
    - Cellular measurements: distribution of different immune cell populations (% and absolute values) in peripheral blood mononuclear cells (PBMCs); activation levels of monocytes and T cells; expression of adhesion molecules
    - Inflammatory cytokines and factors and blood (plasma), including IL-1, HMGB-1, RAGE, sASC, IL-6, etc.
    - Cytotoxicity of peripheral blood mononuclear cells
- Changes in the vaginal, faecal and oral microbiome during the course of the intervention
- Changes in the metabolic profile in urine, faeces and saliva during the course of the intervention
- Changes in epigenetic and functional characteristics of extrinsic skin ageing and skin barrier, their reversion by lifestyle changes and a comparison of both methods for determining the biological age of the skin before and after the intervention (0 and 6 months)
- Change in stem cell fitness of endometrial stem cells from menstrual blood with regard to pluripotency and colony formation potential, etc. (0, 6, 12, 18 and 24 months)
- Change in vascular health factors (pulse wave velocity, intima-media thickness, plaque score) before and after the intervention (0, 6, 12, 18 and 24 months)
- Absolute change in the abdominal fat composition (visceral and subcutaneous)
- Change in EQ-5D-5L scores with regard to health status and quality of life before and after the intervention (0 and 6, 24 and 30 months)
- Change in psychological scale scores before and after the intervention (0 and 6, 24 and 30 months)

- Analysing psychological factors that determine compliance and effectiveness of the intervention with regard to lifestyle changes
- Reprogramming of peripheral blood cells and investigation of epigenetic reprogramming signatures after the intervention

Details on target criteria and recorded values can be found in the relevant sections of the protocol.

3. Study programme & method

3.1. Type of study and summarised overview of the study protocol

The TirolGESUND study is designed as a prospective intervention study. Women between the ages of 30 and 60 who fulfil the inclusion criteria (section 3.2.1) and to whom none of the exclusion criteria apply (section 3.2.2) can participate in the study. A total of 180 women (n=60 for smoking cessation, n=60 for intermittent fasting, n=60 for intermittent fasting with ketogenic supplement) will be included in the study. Recruitment is carried out via written adverts, primarily at Tirol Kliniken GmbH.

Interested participants are invited to an information evening where the study is explained by the study director and questions about the study can be asked. At the end there will be an opportunity to register interest and a preliminary informal screening will be carried out. For practical reasons, interested parties can already take a starter pack with sample containers, which are filled and handed in at the first visit. After a maximum of three weeks, those interested will be contacted and invited to the official screening and baseline visit.

The first visit combines a screening visit and baseline sample collection. Screening takes place via a doctor, who informs the patient again about the study and then obtains written consent. During the first visit, the nutrition arm is also randomised between the two interventions (intermittent fasting, intermittent fasting with ketogenic supplement). The Informed Consent Form is sent to the study coordinator, signed and a copy is sent to the subjects. Clinical, psychological, epidemiological and biochemical baseline values are then measured or baseline samples are collected (cervical smear, blood, urine, stool, oral swab, saliva, optional skin sample, optional menstrual blood sample for pre-menopausal subjects). At the screening visit, the subjects also receive a wearable fitness tracker (Garmin vívosmart® 4), which records baseline factors on general fitness, activity and heart rate (basal and variability) over the course of the study (see section 3.5.10). In the nutrition arm of the TirolGESUND study, participants also receive a FORA® 6 Connect device (section 3.5.10) to record β-hydroxybutyrate in the capillary blood as a marker for ketosis (measurement 3 times a week at the end of the fasting period, i.e. before the first meal).

This is followed over 6 months by a lifestyle intervention (smoking cessation or intermittent fasting +/- ketogenic supplement; both interventions are accompanied by an exercise programme). The interventions are supported by professional counselling and 1:1 coaching. Every two months (months 2, 4 and 6) sample collections of urine, stool, saliva and optional menstrual blood will be organised by the personal coach, and cervical and oral swabs and blood samples will be taken at the study centre. At the dermatology centre, skin biopsies are (optionally) taken at the beginning and end of the study.

In addition, physical activity and general health are continuously recorded during the course of the study using a wearable fitness tracker. After 6 months, clinical, psychological and epidemiological factors as well as health-related quality of life are repeatedly assessed and a final interview is conducted. Schematics of the study procedure are shown in **Illustration *1*** and ***Illustration 2*** (page 22, 23).

At the end of the intervention phase, follow-up visits are carried out at months 12, 18, 24 and 30. The same samples will be taken as during the intervention phase, and adherence to the intervention and, depending on the intervention group, smoking or eating behaviour will be assessed. In addition, an annual sports medicine visit is planned at the sports medicine institute ISAG. If consent is given, the participants sign that the study centre may initially keep their contact details for 7 years for this purpose. This consent can of course be revoked at any time.
Furthermore, the study team is interested in analysing the development of cancer over the next 7 years. For this reason, participants will be asked whether the study team may access their personal data in the localTumour Registry for this period. Due to the prospective setting of the study, any risk factors for the development of a tumour can be identified retrospectively. For this purpose, for a clear assignment the national insurance number of the participants will be requested in consultation with the local Tumour Registry if consent is given.
The subjects are also asked whether they would like to be informed as soon as results from the study can be presented or published. Here, too, the subjects sign a declaration so that the study centre can keep the contact details for this purpose for 7 years.

3.2. Study population

3.2.1. Inclusion criteria

1. Women between the ages of 30 and 60
2. Motivated to change your lifestyle

3. Smoking cessation: currently >10 cigarettes per day and for at least 5 years
4. Intermittent fasting: BMI between 25 and 35

If inclusion criteria 3 and 4 both apply, the subject is assigned to the smoking cessation group.

3.2.2. Exclusion criteria

1. Relevant previous illnesses:
    a. Current or past malignant cancers
    b. Current or past cardiac diseases
    c. Current or past metabolic diseases (e.g. diabetes mellitus type I and II)
    d. Current or past psychiatric illnesses
2. Current pregnancy and breastfeeding period
3. Women with total hysterectomy
4. Women with a known pre-malignant (CIN2/CIN3) or malignant change (invasive cancer) of the cervix (currently and/or in the past)
5. Simultaneous participation in another interventional study

3.2.3. Explanations of inclusion and exclusion criteria

We recruit healthy women who are in an age group for increased cancer risk for women-specific cancers and have risk factors for cancer (smoking status, >10 cigarettes per day for at least 5 years; BMI 25-35), but have no relevant pre-existing conditions. For practical and ethical reasons, we exclude test subjects who are currently pregnant. During screening, a pregnancy test is therefore carried out on women of child-bearing potential (WOCBP). WOCBP are defined as a) premenopausal women, b) women with amenorrhoea (post-menopausal) for less than 12 months, and c) women who have not undergone surgical sterilisation (hysterectomy or bilateral salpingo-oophorectomy).

Intrinsic motivation to change lifestyle is important for compliance with the interventions. This compliance should be further maximised through professional advice and 1:1 coaching.

TirolGESUND – Ernährung

1. Set-up

Inklusion: Frauen im Alter von 30-60 Jahren mit BMI zwischen 25 und 35; motiviert, den Lebensstil umzustellen
Exklusion: Relevante Vorerkrankungen (gegenwärtig oder vergangen): maligne Krebserkrankungen, kardiale Erkrankungen, metabolische Erkrankungen, psychiatrische Erkrankungen; aktuelle Schwangerschaft oder Stillperiode; Teilnahme an einer anderen interventionellen Studie
Rekrutierung: v.a. Frauen, welche in Tirol Kliniken angestellt sind (KH Hall, Schwaz, Innsbruck, Natters, Hoch Zirl)
Fallzahl: 120
Dauer: 6 Monate, optional Nachbeobachtung Monat 12 und 18; 3, 4, 5, 6 und 7 Jahre nach Studienstart

2. Intervention

Intermittierendes Fasten (16:8) +/- ketogenes Supplement (jeweils n = 60)
Diätologische Beratung zur gesunden Ernährung
1:1 Coaching und unterstützendes Bewegungsprogramm
Tragbarer Fitnesstracker und Ketoseüberwachung im kapillären Blut

2. Longitudinale Probensammlung & Datenerfassung

Baseline & Endmessung sowie Follow up-Visiten:

- Gesundheitscheck
- Erfassung der vaskulären Gesundheit
- Hautbiopsie (optional)
- Psychologische Faktoren & Lebensqualität

Alle Visiten:

- Zervixabstrich
- Mundschleimhautabstrich
- Blut-, Harn-, Stuhl- und Speichelprobe
- Menstrualblut (optional)
- Bioelektrische Impedanz

3. Detaillierung der Studienorte

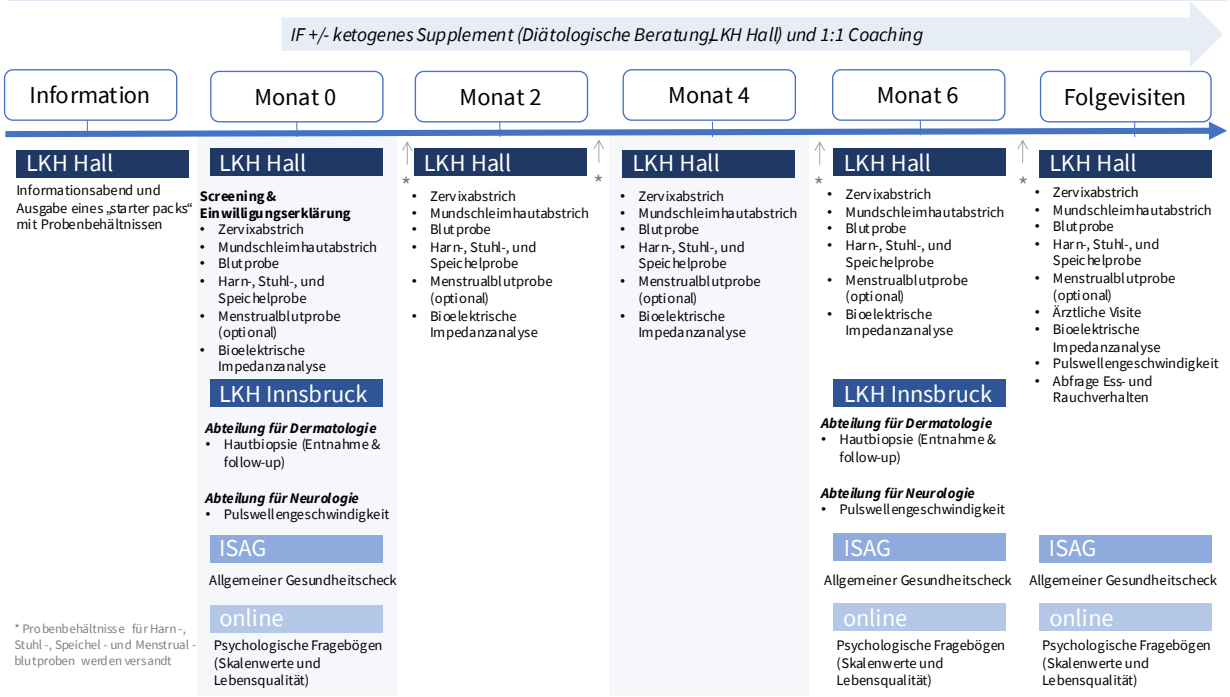

Illustration 1. Scheme of the intervention "Nutrition" (intermittent fasting +/- ketogenic supplement) as part of the TirolGESUND study.

TirolGESUND – Raucherentwöhnung

1. Set-up

Inklusion: Raucherinnen (aktuell > 10 Zigaretten/Tag und seit mindestens 5 Jahren) im Alter von 30 - 60 Jahren; motiviert, den Lebensstil umzustellen
Exklusion: Relevante Vorerkrankungen (gegenwärtig oder vergangen): maligne Krebserkrankungen, kardiale Erkrankungen, metabolische Erkrankungen, psychiatrische Erkrankungen; aktuelle Schwangerschaft oder Stillperiode; Teilnahme an einer anderen interventionellen Studie
Rekrutierung: v.a. Frauen, welche in Tirol Kliniken angestellt sind (KH Hall, Schwaz, Innsbruck, Natters, Hoch Zirl)
Fallzahl: 60
Dauer: 6 Monate, optional Nachbeobachtung Monat 12 und 18 sowie 3, 4, 5, 6 und 7 Jahre nach Studienstart

2. Intervention

Teilnahme an Rauchstopp-Programm (professionelle Suchtberatung)
1:1 Coaching mit unterstützendem Bewegungsprogramm und Ernährungsberatung

2. Longitudinale Probensammlung & Datenerfassung

Baseline & Endmessung sowie Follow up-Visiten:

- Gesundheitscheck
- Erfassung der vaskulären Gesundheit
- Hautbiopsie (optional)
- Psychologische Faktoren & Lebensqualität

Alle Visiten:

- Zervixabstrich
- Mundschleimhautabstrich
- Blut-, Harn-, Stuhl- und Speichelprobe
- Menstrualblut (optional)

3. Detaillierung der Studienorte

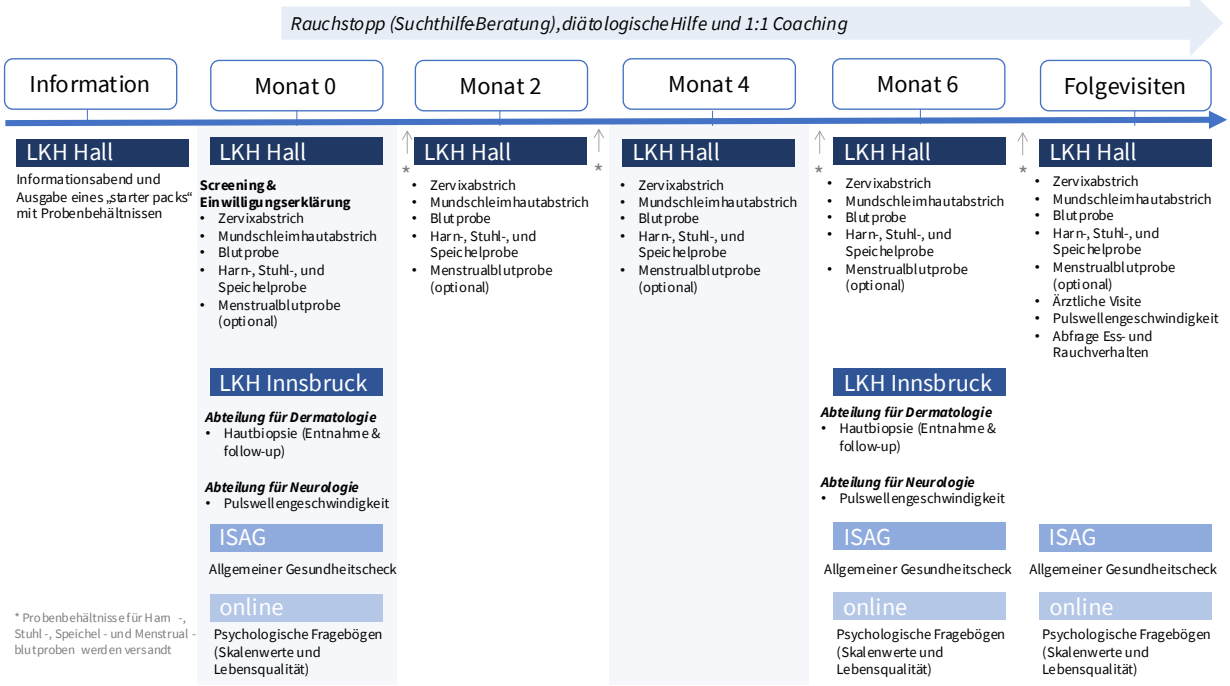

Illustration 2. Scheme of the "Smoking cessation" intervention as part of the TirolGESUND study.

3.3. Recruitment and education

3.3.1. Recruitment

Interested parties will be recruited through the following channels:

- Women who are employed at Tirol Kliniken GmbH (Innsbruck, Hall, Schwaz, Natters and Hochzirl hospitals) and their acquaintances
- Women who attend gynaecological outpatient clinics for routine examinations and meet the inclusion/exclusion criteria.
- Women who were referred to existing smoking cessation programmes

Recruitment is scheduled to begin in Q1 2021; however, invitations/information will be issued/provided in advance.

3.3.2. Information and consent

In addition to the informal information evening, at which the study is explained in more detail, the following is explained by the doctor during the information session and in the written declaration of consent:

- The fact that the study is part of a research project
- The aim and duration of the study
- The interventions and investigations during the study
- The foreseeable risks of study participation and the expected benefits according to the current state of knowledge

It should also be noted that

- Study monitors/auditors/the independent ethics committee and the competent authorities are granted direct access to the subject's original medical records to review the study procedures and data to the extent permitted by applicable law, without violating the confidentiality of the subject's data, and that the subject authorises access to her data by signing a written consent form.
- Records that can be used to identify the test person are treated confidentially and are not made public

Explicit reference is made to the possibility of withdrawing the declaration of consent to the study at any time without giving reasons and without disadvantages for the participants.

The doctor determines whether the person involved has understood the information and is given the opportunity to ask questions. The content of any additional points discussed is noted on the consent form.

The consent of the person involved expressly relates to the collection and processing of health data. Therefore, the participant must be explicitly informed about the purpose and scope of the collection and use of personal data, in particular health data.

3.4. Study-related interventions

In the TirolGESUND study, two study arms will be conducted in parallel:

- Smoking cessation
- Nutrition

  Women in this study arm will be randomised to two interventions:

  - Intermittent fasting
  - Intermittent fasting with a ketogenic supplement

The interventions are described in detail in these Standard Operating Procedures (SOPs) (Appendix A & B for smoking cessation; Appendix C for intermittent fasting). An exercise protocol is also used to support smoking cessation and dietary intervention (see section 3.4.3).

During the course of both interventions, the study participants will receive psychological coaching in order to maximise the effectiveness of the intervention.

3.4.1. Smoking cessation intervention

The smoking cessation intervention (smoke-free programme) is coordinated by academic addiction counsellors and incorporates the latest concepts of motivational and behavioural research (see Appendix A). During 3 appointments and two individual telephone consultations, test subjects learn to better understand their smoking behaviour, to question the motivation that causes them to smoke and to develop coping strategies. The participants are comprehensively prepared by the trainers - certified addiction counsellors, psychologists or psychotherapists - for a fixed stop-smoking day after the third course unit and are then supported and guided by individual coaching (psychology students) to remain smoke-free. In addition, an exercise unit takes place as part of the smoking cessation programme. The content of the exercise unit is related to the theoretical part of the smoking seminar. During this exercise unit, light exercises on breathing, relaxation and rhythm are practised. Finally, there is accompanying dietary support, monitoring of nutritional status (see Appendix B), as well as an exercise protocol for the entire course of the study (see section 3.4.3). Adherence to this intervention is regularly quantified: 1) the coaches regularly ask (once a week) how many cigarettes the test person has smoked within the last 7 days [97] 2) Cotinine in the urine is determined after the visit using an LC-MS measurement.
The intervention includes:

- Three appointments of 180 minutes each (= 2 course units) in a weekly rhythm in a group setting with approx. 6 to 12 participants
- An appointment for exercise
- Two individual telephone consultations per test person
- Additional: exercise protocol and dietary nutritional advice (3.4.3)
- 1:1 Coaching

3.4.2. Intermittent fasting (with/without ketogenic supplement)

The "Intermittent Fasting" intervention is carried out as "Time Restricted Feeding" based on the work published by de Cabo and Mattson [65]. It concerns a nutritional therapeutic intervention and is carried out by introducing a 16:8 intermittent fasting (IF) regime in the following way (see also Appendix C):

1. Week: 10 hours of food intake, 14 hours off food for 5 days per week
2. Week: 8 hours of food intake, 16 hours off food for 5 days per week
3. Week and subsequent weeks: 8 hours of food intake, 16 hours off food for 7 days per week
4. Week and subsequent weeks: 6 hours of food intake, 18 hours off food for 7 days per week (optional)

In addition, one group will be given a ketogenic supplement to reinforce ketosis for the entire duration of the study. This is done once a day before the start of the fasting phase. After the introductory phase, which extends over the first month, a daily fasting interval of 16 hours should be maintained for the remaining study duration of approx. 5 months. An optional extension to an 18-hour fasting phase is possible. The timing of the fasting phases is left to the subjects themselves. As part of the nutritional counselling at the start of the study, the subjects will be advised on the practical implementation of the IF regime based on their previous diet (according to the dietary protocol). Exemplary daily plans for achieving the specified fasting phases are discussed. The counselling sessions after 2 and 4 months serve to monitor dietary progress and motivate the continuation of the TRF regime. The diet is analysed using a dietary protocol (see Appendix D). Using the FORA® 6 Connect device, the β-hydroxyburyrate levels in the capillary blood are recorded at home 3 times a week. After 6 months, counselling is provided regarding the gradual termination of the TRF regime in reverse order to the introduction and continuation of a balanced mixed diet or, in the case of an optional study extension, the ongoing dietary support of the TRF regime. In addition to counselling appointments after 2 and 4 months by clinical dieticians, the intervention is supported by psychological coaching and a supportive exercise protocol. For the follow-up visits, the DEGS nutrition questionnaire (see Appendix E) is issued to assess nutritional behaviour.

3.4.3. Adjuvant exercise programme

Based on the health and fitness check at the sports medicine institute ISAG (see 3.5.2), the study participants in both interventions receive recommendations for a supportive exercise programme. In addition, the test subjects are provided with exemplary training suggestions for the areas of endurance, strength and flexibility. In order to maintain compliance, up to 3 guided exercise sessions will take place in the group.

The type, scope and intensity of physical activity during the study period are recorded once a month using a questionnaire (International Physical Activity Questionnaire - IPAQ; see questionnaire in Appendix F). which records the respective physical activity parameters retrospectively for the last week before completion.

3.5. Study visits and examinations

In the course of the studies, several longitudinal examinations and sample collections will take place (for a temporal description of the individual examinations, see **Table 1**. The details of the examinations are described in the following sections. **Table 2** provides details on the respective biomaterials.

Table 1. Visit plan.

	Screening	Lifestyle intervention & observation			Follow up			
		Month 2	Month 4	Month 6 (endpoint)	Month 12 (optional)	Month 18 (optional)	Month 24 (optional)	Month 30 (optional)
Time (week) **Time window (+- weeks)**	**0** **-**	**8** **7-10**	**17** **16-19**	**26** **25-28**	**52** **50-55**	**78** **75-79**	**104** **101-105**	**130** **127-131**
Declaration of consent	x							
Inclusion/exclusion criteria	x							
Pregnancy test	x							
Epidemiological questionnaire	x							
Study visit (swabs & sample delivery):	x	x	x	x	x	x	x	x
Cervical smear	x	x	x	x	x	x	x	x
Oral mucosa swab	x	x	x	x	x	x	x	x
Blood sampling	x	x	x	x	x	x	x	x
Urine sample (native)	x	x	x	x	x	x	x	x
Saliva sample (native and stabilised)	x	x	x	x	x	x	x	x
Stool sample (native and stabilised)	x	x	x	x	x	x	x	x
Menstrual blood sample (native and stabilised) [a]	x	x	x	x	x	x	x	x
Recording of adverse events [c]	x	x	x	x	x	x	x	x
Bioelectrical impedance analysis [b]	x	x	x	x			x	x
General health check including medical history, physical examination, exercise ECG and laboratory test	x			x			x	x
Vascular health	x			x			x	x
Skin biopsy (optional)	x			x				
Psychological questionnaires	x			x			x	x
IPAQ questionnaire	x	x	x	x	x	x	x	x
Nutrition questionnaire							x	x

[a] only for premenopausal women who are having their period

[b] only in the nutritional arm of the study

[c] Subjects can also report adverse events in connection with the study intervention to the study centre at any time

Location legend: LKH Hall; ISAG Natters; LKH Innsbruck/MUI; Online

3.5.1. Screening and baseline visit

Interested women who fulfil the inclusion and exclusion criteria according to their own information are invited to the hospital of Hall in Tirol. The investigator will carefully check all inclusion/exclusion criteria. A pregnancy test is carried out to rule out pregnancy. Detailed information about the study is provided again and written consent to participate in the study is obtained.

For practical reasons, the baseline samples are already taken during this visit after the declaration of consent (containers for collecting urine, faeces, saliva and menstrual blood samples are already given to the test subjects on the informal information evening). Furthermore, subjects receive the fitness tracker and their FORA® 6 Connect device at this time (the latter only for subjects in the nutrition arm of the study from the time of this amendment, V4; reasons for change in section 3.7). A detailed explanation of mobile devices can be found in Section 3.5.10. For practical reasons, the screening and baseline visit and further cervical smear samples are taken at a time when the proband is not menstruating.

In the nutrition arm of the study, the test subjects are also randomised between the two interventions within a week.

3.5.2. Health and fitness check

The health check includes the following examinations:

- Medical history
- Anthropometry: height, body weight, body mass index
- Electrocardiogram (ECG) and blood pressure measurement at rest
- Minor spirometry
- Basic laboratory (venous blood sampling and urinalysis)
- Endurance ergometry on the bike
- Results discussion

3.5.3. Sample collection - cervical smear, oral swab, blood, urine, faeces and saliva

The samples are taken directly during the study visits at LKH Hall, or the relevant containers are provided and handed in during the study visit. The samples are primarily processed in the laboratory at the hospital of Hall in Tirol as described below. Until further use, the samples are stored in a cooling device designated for the TirolGESUND study. Access to this unit is only possible with a key and is restricted to laboratory staff involved in the study.

3.5.3.1. Cervical smear:

Collection: The smears are taken at the gynaecological outpatient clinic in the hospital of Hall in Tirol. After vaginal positioning with the specula and visualisation of the portio, the smear is taken using a brush with multiple rotations and immersion in the BD SurePath Collection Vial 10ml (Becton, Dickinson and Company, Sparks MD 21152 USA) or ThinPrep® Collection Kit (Hologic).

Sample processing: The container is brought to the LKH Hall laboratory and processed within 6 hours; the sediment at the bottom of the container after 1 hour is collected with a pipette, transferred to a cryo-vial and centrifuged at 2,500 rpm for 10 minutes. The supernatant is then removed.

Sample storage: The pellet is stored at -80°C.

Planned analyses: DNA methylation analyses and analysis of somatic mutations; Mirobiome analyses

3.5.3.2. Oral mucosal swab:

Collection: The oral mucosal swabs are taken during the visit for cervical smears (see section 3.5.3.1) at the gynaecological outpatient clinic in the hospital of Hall in Tirol. The swab (Whatman Foam Applicator Swab) is swabbed 10 times per cheek side on the oral mucosa (at least 10 s) and pressed onto the Whatman FTA Elute Indicating Filter Paper.

Sample processing: The container is dried at room temperature for 3 h and then stored in a sterile and airtight container at room temperature. DNA is extracted using an elution protocol.

Sample storage: The sample container is **stored** at room temperature (dried) until DNA extraction.

Planned analyses: DNA methylation analyses and analysis of somatic mutations

3.5.3.3. Blood sample:

Collection: Blood samples are taken as part of the cervical smear test (see section 3.5.3.1) at the hospital of Hall in Tirol when the samples are handed in. A total of 37 ml of blood is taken, of which 2.5 ml is taken in PAXgene blood DNA tubes (BD Biosciences, #761165) for DNA analysis, 30 ml in Vacuette® 9 ml NH Sodium Heparin tubes (Greiner, #455051) for PBMC and plasma extraction, and 0.5 ml in Sarsted S-Monovette® Serum tubes (Sarstedt, #06.1663.001), and 4 ml for routine diagnostics (blood count, CRP). The samples are stored at room temperature until further processing (maximum 20 h).

Sample processing: PAXgene blood DNA tubes are stored at -20°C until DNA extraction. Serum tubes are stored for at least 30 minutes, centrifuged and frozen at -20°C. Samples

for PBMC and plasma extraction are stored at room temperature; PBMC and plasma are separated by Ficoll density gradient centrifugation. In a 50 ml Falcon tube, 13.33 ml Ficoll-paque (Lymphoprep™, Stem Cell Technologies # 07861) is placed and carefully overlaid with 20 ml anticoagulated whole blood. The Falcon tubes are centrifuged for 25 min at 2,200 rpm (without brake). The plasma (top layer) is removed and frozen in 1 ml aliquots at -80°C. The PBMCs are carefully removed with a Pasteur pipette, transferred to a fresh 50 ml Falcon tube and washed with RPMI by first filling the tube and then centrifuging at 300 x g for 10 minutes. The cell pellet is washed again with RPMI and 10% FCS. Finally, the cell pellet is resuspended in 5 ml RPMI and 10% FCS and the cell concentration is determined. The cells are then centrifuged again and resuspended to a cell concentration of approx. 1-2 $x10^7$. The cells are mixed with an equal volume of 2x freezing mix (20% DMSO in FCS) and transferred in 1 ml aliquots into cryovials on ice. The cryovials are immediately transferred to a "Mr Frosty" container and frozen at -80°C. After 24-48 h, the PBMCs are transferred to liquid nitrogen containers for long-term storage and later experiments.

Sample storage: PAXgene blood DNA tubes are stored at -20°C until DNA extraction. Plasma is stored at -80°C. Isolated PBMCs are stored in liquid nitrogen at -196°C at the Institute for Biomedical Ageing Research.

Planned analyses: lipids, HbA1c, CRP; DNA methylation analyses and CHIP analysis; single cell sequencing (RNA + ATAC-Seq); cytotoxicity assay; cellular factors from PBMCs: General immunological profile, including macrophage, NK, and T cell populations, activation, adhesion molecule expression, functional assays (cytokine profile after activation of T cells and monocytes); Plasma factors: broad cytokine profile and oxidative stress biomarkers; Metabolome; Reprogramming.

3.5.3.4. Saliva sample:

Collection: Prior to the study visit at the EUTOPS Institute, a sterile container (Sarstedt Salivette, #51.1534) and cold packs are given to the test subjects. The container is filled and, together with urine and faeces containers, delivered to the laboratory at the hospital of Hall in Tirol. Samples (collected according to Appendix G) are stored at 4°C until processing. To determine the oral microbiome and metatranscriptome, another saliva sample is collected in a DNA/RNA-stabilising container (see SOP in Appendix I).

Sample processing: The saliva samples for metabolic profiling are centrifuged after delivery (2000 x *g* for 10 min at 4°C) and the supernatant is transferred to fresh containers.

Sample storage: The supernatant of the native saliva samples (metabolic profile) is stored at -80°C. For determination of the oral microbiome and metatranscriptome, samples are stored at -80°C until DNA/RNA extraction.

Planned analyses: metabolic profile, oral microbiome and metatranscriptome

3.5.3.5. Urine sample:

Collection: Before the study visit, a sterile container (Sarstedt Urine Monovette® 10 ml, #10.252) and cold packs are given to the subjects. The container is filled with the first morning urine (smoking cessation intervention) or with the last urine before food intake (see SOP in Appendix H) and delivered to the laboratory of the LKH Hall together with saliva and stool containers. Samples are stored at 4°C for a maximum of 8 hours until processing.

Sample processing: The samples are centrifuged after delivery (2000 x *g* for 10 min at 4 degrees). The supernatant is transferred to fresh containers.

Sample storage: The supernatant is stored at -80°C.

Planned analyses: Metabolic profile

3.5.3.6. Stool sample:

Collection: Before the study visit, two containers are given to the test subjects. Both native faecal samples (approx. 5 g in Sarstedt Fecal Collection Tubes, 80.734.301) and stabilised faecal samples (in Zymo DNA/RNA Shield Fecal Collection tube, Zymo #R1101) are collected on the following day (SOP in Appendix I) and handed in together with saliva and urine samples on the day of the study visit in the laboratory of the LKH Hall. Where possible, native faecal samples should be stored at 4°C between collection and delivery but can be transported at room temperature.

Sample processing: For both native and stabilised faecal samples, the samples are aliquoted 3 times 100 mg each and frozen at -80°C.

Sample storage: Aliquots of native and stabilised faecal samples are stored at -80°C.

Planned analyses: faecal metabolome, microbiome and metatranscriptome

3.5.4. Survey of psychological factors

Location: Online questionnaire

Determined values:

- *Questionnaire scores on (1) general self-efficacy expectation, (2) specific self-efficacy expectation regarding nicotine abstinence, (3) health-related control beliefs and (4) risk culture beliefs about health*

- *Quality of life via EQ-5D-5L*

Self-efficacy expectation is understood as the conviction that one can perform desired behaviours or cope with challenging situations on the basis of one's own competencies. General, i.e. cross-situational, self-efficacy expectation is measured using the *General Self-Efficacy Expectation Scale.* [98]. The one-dimensional scale contains 10 items to be answered on 4-point Likert scales ("strongly disagree" to "strongly agree"). The *self-efficacy scale for smoking* cessation is also presented as an area-specific instrument [99]. The one-dimensional questionnaire contains 9 items (5-point Likert scales, "not at all confident" to "extremely confident") to assess the ability to resist smoking in positive social situations, stressful situations and habitual temptation situations.

Health-related control beliefs imply the assessment of the extent to which one's own state of health can be influenced by oneself (internal control) or is determined by forces outside oneself (external control). The construct is quantified using the *questionnaire for surveying control beliefs about illness and health* [100] . The three-dimensional instrument (21 items, 6-point Likert scales, "strongly disagree" to "strongly agree") provides the following scales: (1) internality (conviction that health can be controlled by oneself), (2) social externality (conviction that health can be controlled by other people such as doctors or carers) and (3) fatalistic externality (conviction that health is dependent on chance or fate).

In addition to individual beliefs, risk perception and behaviour is also largely determined by culture-specific beliefs, so-called risk cultures. The *health-related risk culture* [101] measures the conviction of how relevant individual influences are for health based on the three dimensions of person, social context and risk situation and the two levels of observable and not directly observable, resulting in six factors: (1) Person observable (40 items), (2) Person non-observable (39 items), (3) Context observable (26 items), (4) Context non-observable (27 items), (5) Risk observable (30 items), and (6) Risk non-observable (21 items) (for all items visual analogue scale from 0 to 100).

Participants receive an individualised link to an online questionnaire (Qualtrics for psychological scale values or Askimed or subsequently developed data entry masks for EQ-5D-5L). The data entered is processed anonymously and analysed in aggregated form. Questionnaires can be found in Appendix Q.

3.5.5. Assessment of the range of motion using IPAQ

The International Physical Activity Questionnaire (IPAQ)[102] comprises a compilation of 4 questionnaires. Long (5 activity domains surveyed independently) and short (4 general items) versions are available for both telephone and self-administered methods. The intention of the questionnaires is to provide simple instruments that can be used to obtain internationally comparable data on health-promoting physical activity. Details can be found in Appendix F.

3.5.6. Survey of nutritional behaviour using the DEGS nutrition questionnaire

The DEGS nutrition questionnaire (Study on the Health of Adults in Germany) is a food frequency questionnaire (food frequency questionnaire) [103]. It asks about the frequency of consumption and the usual portion sizes of 53 food groups consumed in the last four weeks. Details can be found in Appendix E.

3.5.7. Collection of relevant information for the follow-up visits

Appendix R contains the questionnaires to be completed at the follow-up visits in order to document the visit and the samples collected and, depending on the intervention arm, to ask about eating and smoking behaviour after the end of the active intervention.

3.5.8. Bioelectrical impedance analysis, body length and body weight (nutrition study arm)

Location: hospital of Hall in Tirol, EUTOPS Headquarters

Determined values:

- *Body length*
- *Body weight*
- *Bioimpedance measurement*

This analysis is only performed in the "Nutrition" study arm. The SOP for this analysis can be found in Appendix J. Body length and body weight are recorded using standard methods. Bioelectrical impedance measurement is determined using the BIA Corpus RX4000M. The last large main meal should have been 2-3 h ago, fluid intake should have been paused 0.5-1 h before the measurement, and the bladder should have been emptied before the measurement. In addition, no sporting activity should be carried out for 8 hours before the measurement. Test subjects should lie flat on a patient couch for 5-10 minutes before the measurement. The measurement data is entered in the form provided for this purpose (Appendix K).

3.5.9. Vascular health and visceral abdominal fat

Location: Department of Neurology (Medical University of Innsbruck) and hospital of Hall in Tirol

Determined values:

- *Pulse wave velocity (carotidofemoral)*
- *Intima-media thickness of the common and internal carotid artery on both sides*
- *Plaque score of the common carotid artery and internal carotid artery on both sides*
- *Visceral and subcutaneous abdominal fat*

This examination will take place at the beginning and at the end of the study, in accordance with the SOP described in Appendix J. Up to 10 patients can be examined in one afternoon. The pulse wave velocity is performed in a supine position after a resting period of at least 5 minutes, with head and shoulders elevated 30 degrees.
Visceral and subcutaneous abdominal fat are measured using an adapted standard ultrasound measurement according to Pontiroli et al. [104]. A linear 10 MHz transducer is placed at the xipho-umbilical line near the belly button and visceral fat is measured after uniform expiration. Visceral fat is measured from the internal surface of the rectus abdominis muscle to the aortic wall, excluding the thickness of the muscle and skin from the measurement. The measurements are performed in triplicates. The mean value of these three measurements is calculated and used for further evaluation.

3.5.10. Mobile devices for continuous data collection

As part of the study, test subjects receive a mobile fitness tracker (Garmin® Vivosmart 4; see Appendix M), which is used to collect continuous data on exercise behaviour and general health (e.g. number of steps, calories burned, basal heart rate, heart rate variability in an inactive state ("stress"), sleep quality, "body battery"). An account with the study number and without personal data is created for each participant. Data is collected via the Garmin Connect™ app by pairing the device with the participant's smartphone via Bluetooth. By accepting the fitness tracker, participants agree to Garmin's terms and conditions. The study management has a contract with Garmin Health API, which allows certain parameters of our test subjects to be downloaded in advance via API to our secure data storage, where this data is stored for analysis as part of the study.

In the nutrition arm of the study, the test subjects are also given a device that measures the β-hydroxybutyrate levels in the capillary blood and can thus indicate the onset of ketosis (see Appendix O). The measurement should take place three times a week at the end of the fasting period for the duration of the study.

These devices are primarily used to record continuous data and to assess the compliance of the test subjects with regard to exercise and diet. At the same time, however, these devices also serve as motivation and "biofeedback" for the test subjects. Both devices can be kept by the test subjects after the study has been completed.

Test subjects receive help in operating the fitness trackers and FORA® 6 Connect devices from coaches and other members of the study team.

Measured values:

- Fitness tracker:
    - Step count
    - Calorie consumption
    - Heart rate variability
    - Sleep quality
    - "Body battery"
    - Active minutes per day
    - VO_2 with movement
- FORA 6 Connect
    - β-hydroxybutyrate in capillary blood

3.5.11. Skin biopsies (optional)

Location: Department of Dermatology (Medical University of Innsbruck)

Sampling: Skin biopsies and barrier measurements are taken on the interior of the extremities in accordance with the SOP in Appendix P. The samples are taken with a separate declaration of consent at the Department of Dermatology of the Medical University of Innsbruck.

Sample processing: Immediately after taking the skin biopsies, biopsy material is further processed in the dermatology department as follows:

- Part of the biopsy is stored at -80°C for lipid analyses using LC-MS/MS.
- Part of the biopsy is pre-fixed in Karnovsky buffer at room temperature for ultrastructural analyses and sent for further sample preparation.
- Part of the biopsy is FFPE fixed, and the blocks are stored at RT until analysed
- Part of the biopsy is used to obtain RNA and proteins; further storage until analysis takes place in liquid nitrogen (RNA) or at -80°C (protein lysates)

For analyses at the Institute of Molecular Biology (IMB; Edenhofer) and the the Research Institute for Biomedical Ageing Research (IBA; Jansen-Dürr), biopsy material is transferred into transport tubes (Sterilin™ Tubes with Screw Cap, 5ML; ThermoFisher Scientific #Z5PE), in which 2 ml cold (4°C) transport medium (DMEM (Sigma Aldrich, #D5546) with 10% FBS (Thermofisher Scientific #10270106), 5 µg/mL Amphotericin B (Sigma Aldrich #A2942), 1% Pen/Strep (Sigma Aldrich #P433) and 100 µg/mL Gentamicin (Sigma Aldrich #G1397)) was placed. This allows the tissue to be kept alive for a limited time (minimum 24 h to approx. 1 week).

Transport containers with biopsy material are immediately transferred on ice from the dermatology department to the IMB or IBA and processed within 30 minutes.

To obtain induced pluripotent stem cells (iPSCs), primary fibroblast cultures are first produced from the biopsy material at the IMB as follows:

- Incubation with Dispase II (2.4 U/ ml; ThermoFisher # 17105041) for 16 hours at 4 °C
- Manual removal of the epidermis using tweezers
- Treatment with 2 ml collagenase type 2 (500 U/ml CDU; ThermoFisher #17101015) in a 15 ml FALCON tube for 45 min at 37 °C
- Centrifugation for 5 min at 180 x g and resuspension of the pellet in 10 ml DMEM-FCS-Gentamicin medium
- Centrifuge and resuspend again, then transfer to T25 tissue culture flask (ThermoFisher #156367) and incubate at 37 °C in 5% CO2 atmosphere.

The "Sendai Virus infection kit" is used to generate iPSZ (CytoTune™-iPS2.0 Sendai Reprogramming Kit, ThermoFisher # A16517). The fibroblasts are detached from the adherent phase by treatment with 2.5 ml trypsin/EDTA (0.05%; Gibco # 25300096) for 5 min at 37 °C (5% CO2) and transferred to a 15 ml FALCON tube by resuspending with 2.5 ml of fibroblast medium (DMEM/10% FCS). After centrifugation (180 x g for 5 min), the cell pellet is resuspended in 1 ml of fibroblast medium and the cell count is determined using a Neubauer cell counting chamber. For each iPSZ preparation, 3 x 104 cells are seeded per well of a 24 well tissue culture plate and incubated overnight at 37 °C, 5% CO2. The next day, a virus aliquot (one aliquot is sufficient for the infection of 10 preparations) is thawed and resuspended in 1 ml DMEM/10% FCS. The viruses are added to the cells at an MOI of 3 and incubated overnight at 37 °C and 5% CO2. On the following day, the cells are switched to virus-free fibroblast medium and cultivated for a further six days (daily medium change). On day 7 post inf. the cells are detached and placed at a density of 1 × 105 on previously prepared cells plated with murine embryonic fibroblasts (Merck #PMEF-N) (well of a 6-well plate). 24 hours later, the culture is switched to iPSC medium (StemMACS™ iPS-Brew XF, Miltenyi Biotec. # 130-104-368) and cultivated with daily medium changes. iPSZ colonies are manually isolated after 3-4 weeks and expanded in iPSC medium.

Sample storage:

- Parts of the biopsy for lipid analyses (using LC-MS/MS): Storage at -80°C.
- Parts of the biopsy for the extraction of RNA and proteins: Storage in liquid nitrogen (RNA) or at -80°C (protein lysates)
- Parts of the biopsy for ultrastructural analyses: pre-fixed at room temperature in Karnovsky buffer and sent for further sample preparation.

- Another part of the biopsy is FFPE-fixed, and the blocks are stored at RT until analysed

Planned analyses:

- *DNA & RNA extraction*
- *Fibroblast culture and generation of induced pluripotent stem cells*
- *Skin barrier parameters, lipid analyses*
- *Immunohistochemistry & -fluorescence*

3.5.12. Menstrual blood samples (optional and exploratory)

Location: hospital of Hall in Tirol, EUTOPS Headquarters

Collection: Menstrual blood samples are collected in two forms: fixed (for analysing DNA and DNA methylation) and non-fixed (for cell culture experiments on the "fitness" of stem cells - exploratory). These samples are only collected from menstruating premenopausal women.

Fixed samples: We are mainly interested in the fixed collection of menstrual blood samples. To obtain endometrial stem cell DNA from menstrual blood, premenopausal subjects who consent to optional menstrual blood collection will be provided with a menstrual cup and a container for sample storage at the beginning of the study (BD SurePath). Menstrual blood on the second day of menses is then collected with the menstrual cup and stored at 4°C until sample collection at the study visit.

Non-fixed samples: For exploratory purposes we are also interested in non-fixed menstrual blood samples to perform functional stem cell assays. In this case, we estimate at the beginning of the study when menses will occur. Each month, the subjects are then provided with a 50 ml container filled with PBS buffer containing 250 mg/l amphotericin B, 100 mg/l streptomycin, 100U/ml penicillin and 2 mM EDTA-Na^{2+}. The unfilled container can be stored in the refrigerator for a maximum of 1 week. On the second day of menses, the menstrual blood is collected via the menstrual cup and transferred to the container with PBS and supplementation. The container can be stored in the refrigerator for a maximum of 2 days until it is handed in at the hospital of Hall in Tirol.

Sample processing:

Fixed samples: For fixed samples, a pellet is obtained and stored at -80°C, similar to cervical smear samples.

Non-fixed samples: Non-fixed samples are couriered to the Institute of Molecular Biology (Laboratory for Genomics, Stem Cell Biology and Regenerative Medicine) and separated by Ficoll density gradient centrifugation (10 minutes) [105]. The cells in the buffy coat are transferred to a fresh container, washed twice with PBS and resuspended in growth medium (DMEM with high glucose (Hyclone, USA), supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin). The cells are kept in liquid culture for 72 h (25 cm^2 cell culture flask, 37°C, 5% CO_2). Non-adherent

cells are washed and adherent cells (menstrual blood-derived stem cells, menSCs) are further cultured and kept for analyses and storage.

Sample storage:

Fixed samples: Pellet at -80°C until DNA extraction

Non-fixed samples: Cryopreservation in liquid nitrogen (< -135°C) at the Institute of Molecular Biology

Planned analyses:

Fixed samples:

- DNA methylation and mutation load

Non-fixed samples (depending on the yield and effectiveness of the protocol):

- Functional assays that determine stem cell fitness (non-fixed cells):
    - Colony formation potential
    - Differentiation potential
    - Immunoassays
    - Reprogramming

Table 2. Details of biomaterials obtained (place of collection, type of sample, collection vessel, transport and storage conditions and values determined).

	Cervical smear	Blood sample	Stool sample	Oral mucosa swab	Saliva	Urine	Skin biopsy	Menstrual blood
Place of collection	Gynaecological outpatient clinic, hospital of Hall in Tirol	Laboratory of the hospital of Hall in Tiro	At home of test persons, delivery to the laboratory of the hospital of Hall in Tiro	Hospital of Hall in Tiro	At home of test persons, delivery to the laboratory of the hospital of Hall in Tiro	At home of test persons, delivery to the laboratory of the hospital of Hall in Tiro	Dermatology, Venereology and Allergy (MUI)	At home of test persons, delivery to the laboratory of the hospital of Hall in Tiro
Type of sample	Liquid-based cytology	21 ml peripheral whole blood	Native and stabilised faecal sample	Oral mucosa swab	2 ml saliva	2 ml first morning urine (midstream urine)	6-8 mm biopsy, inside of the upper arm	5-10 ml menstrual blood (2nd day of menses)
Collection vessel	BD SurePath Collection Vial 10 ml or Hologic Thinprep® Collection kit	PAX gene DNA blood tubes (2.5 ml) Vacuette® 9 ml NH Sodium Heparin tubes (Greiner, #455051) (18 ml) Sarsted S-Monovette ® Serum tubes (Sarstedt, #06.1663.001) (0.5 ml)	Sarstedt® Fecal collection Tubes (native), Zymo DNA/RNA Shield fecal collection tube (stabilised)	4N6FLOQ Buccal Swabs	Sarstedt Salivette® (#51.1534) (native) Zymo DNA/RNA Shield saliva collection tube (stabilised)	Sarstedt Urine Monovette® 10 ml (10.252)	1) Sterilin™ Tubes with Screw Cap, 5ML ThermoFisher Scientific #Z5PE, 2 mL DMEM 2) Formalin 3) Karnovsky buffer	Menstrual cup, then transfer to BD SurePath (fixed) PBS with supplementation (amphotericin B, penicillin, streptomycin) (not fixed)
Transport conditions	4°C	Room temperature	Native: 4°C until delivery, RT Transport Stabilised: up to 4 weeks at RT (until delivery)	Room temperature	Native: 4°C for a maximum of 8 h (metabolome) Stabilised: up to 4 weeks at RT (until release - microbiome, metatranscriptome)	4°C	1) 4°C for a maximum of 24 h 2+3) Room temperature	4°C
Storage	Laboratory of the hospital of Hall in Tirol (EUTOPS cooling unit), -80°C	Laboratory of the hospital Hall in Tirol (EUTOPS cooling unit), Pax gene DNA blood tubes/DNA, -20°C PBMCs, -196°C Plasma, -80 °C Serum, -80 °C	Laboratory of the hospital of Hall in Tirol Native: aliquoting (n=3, 100 mg each), -80°C Stabilised: Aliquoting (n=3), storage -20 or -80°C	Laboratory of the hospital of Hall in Tirol (EUTOPS cooling unit), -20°C	Laboratory of the hospital of Hall in Tirol Native: -80 °C (metabolome) Stabilised: Aliquoting (n=2), storage -20 or -80°C	Laboratory of the hospital of Hall in Tirol, -80°C	1) IMB/Derma -80°C 2) Formalin 4°C 3) Glutaraldehyde 4°C	Fixed: Laboratory of the hospital of Hall in Tirol (EUTOPS cooling unit), -80°C Not fixed: Cultivation at University of Innsbruck; < -135°C
Determined values	DNA extraction • DNA methylation • Genome sequencing Cervical microbiome	DNA extraction: • DNA methylation • Genome sequencing Cellular: • Immune populations • Cell activation (Th, macrophages) Serum/plasma: • Cytokine profile • Metabolome • Reprogramming	Native: • Metabolome Stabilised: • Microbiome • Metatranscriptome	DNA extraction • DNA methylation • Genome sequencing • (possibly microbiome, metatranscriptome, oral microbiome)	Native: • Metabolome Stabilised: • Microbiome • Metatranscriptome	• Metabolome • Cotinine (only for smoking cessation)	1) iPSZ production, DNA methylation, transcriptome, IHC: RNAseq analysis Protein analysis by Western blot, IHC, IF for proteins regulating differentiation, mitochondrial function and autophagy; 2) Lipid profiles by HPLC-MS/MS analysis; 3) TEM for lipids and organelles	DNA extraction (fixed) • DNA methylation and mutation load Cultivation (not fixed): Potential of stem cells: • Colony formation potential • Differentiation potential • Reprogramming • IHC • Migration potential

3.6. Expected duration of study

The study is planned for a total period of 36 months. The main study will run for 30 months per subject; the main endpoint (completion of the active intervention) is 6 months, with the option of a follow-up 12, 18, 24 and 30 months after the start of the study if study participants agree to participate.

- "First participant in": Q1 2021
- Inclusion of test subjects: continuously during the study (dynamic cohort)
- Duration of the study per subject: 30 months (minimum 6 months, optional follow-ups 12, 18, 24 and 30 months)
- "Last participant out": Q2 2024

3.7. Switching from Ketonix to FORA® 6 Connect for measuring ketosis

In previous versions of the study protocol (1-3), subjects were offered a mobile Ketonix device to assess metabolic change (instructions for use **Error! Reference source not found.**), which was intended to measure acetone in the breath. However, there were problems with the daily use of the devices and the measured values were inconsistent. Unfortunately, the devices dampened the motivation of the test subjects. After a more detailed literature review, the study management decided that the FORA® 6 Connect device should be offered instead (instructions for use in Appendix **Error! Reference source not found.**). Similar devices are already used in routine glucose and ketosis measurement for diabetics and a recently published study also shows that nutritional ketosis, e.g. due to hypocaloric diet, can also be detected by capillary blood measurement [106].

4. Collected biomaterial and archiving

Table 4 lists the samples collected over the duration of the study. For optional follow-up (months 12, 18, 24 and 30), cervical and oral mucosal swabs as well as blood, urine, faeces, saliva and menstrual blood samples are collected.

Table 3. Summary of the biomaterials collected in the TirolGESUND study.

Basic measurement (month 0)	Month 2	Month 4	Final measurement (month 6)	Control visits
Cervical smear	Cervical smear	Cervical smear	Cervical smear	Cervical smear
Blood sampling	Blood sampling	Blood sampling	Blood sampling	Blood sampling
Oral mucosal swab	Oral mucosal swab	Oral mucosal swab	Oral mucosal swab	Oral mucosal swab
Urine sample	Urine sample	Urine sample	Urine sample	Urine sample
Stool samples (native, stabilised)	Stool samples (native, stabilised)	Stool samples (native, stabilised)	Stool samples (native, stabilised)	Stool samples (native, stabilised)
Saliva samples (native, stabilised)	Saliva samples (native, stabilised)	Saliva samples (native, stabilised)	Saliva samples (native, stabilised)	Saliva samples (native, stabilised)
Menstrual blood samples (optional; fixed, exploratory non-fixed)	Menstrual blood samples (optional; fixed, exploratory non-fixed)	Menstrual blood samples (optional; fixed, exploratory non-fixed)	Menstrual blood samples (optional; fixed, exploratory non-fixed)	Menstrual blood samples (optional; fixed, exploratory non-fixed)
Skin biopsy			Skin biopsy	Skin biopsy

All biomaterials will be archived in the International Biobank Zams after completion of the study (or early termination of the study). For organisation and overview of collected samples we will use tube barcoding in combination with a laboratory information management system (LIMS system). The data will be archived for further studies in research and development in the context of cancer and prevention research and it is expected that the materials will continue to be used for up to 20 years. For further research projects with these samples outside the described study, a further application will be submitted to the ethics committee.

5. Effect, benefits and possible risks

The study primarily serves as a research project to prove that lifestyle interventions can contribute to a measurable risk reduction for women-specific cancers; this risk reduction is determined via DNA methylation in cervical cells as a surrogate marker. The knowledge gained from this study will potentially guide future studies and patient treatment in the context of oncology prevention. There is also an expected benefit to the subjects through adoption of a healthier lifestyle and associated

normalisation of various values (inflammatory factors, oxidative stress, and - in a broader sense - cancer risk reduction), although the extent of individual risk reduction will only be determined by the study.
No risks are foreseen for the planned interventions or examinations. The following minor side effects may occur: Spotting after cervical smear, pain and/or bruising during blood sampling. In the diet arm of the study, side effects of the change in diet or intermittent fasting may also occur (feeling hungry, digestive problems). These symptoms will be explained in the informed consent form.

5.1. Recording of adverse events (Adverse Events)

Adverse events (AEs) and serious adverse events (SAEs) are defined as all adverse changes from the subject's baseline condition. These include abnormal laboratory reports outside the reference range, symptoms or illnesses that are considered clinically significant by the treating investigator. These include:

- The worsening or increase in frequency and/or intensity of existing medical conditions
- Clinically significant laboratory tests outside the reference range

Do not belong to AE/SAEs:

- Planned interventions and hospitalisations
- Planned medical or surgical procedures, for example operations, endoscopic examinations, tooth extractions, blood transfusions
- Pre-existing illnesses (outside the inclusion/exclusion criteria) or medical conditions that do not worsen

AEs/SAEs are not to be expected due to the inclusion of "healthy" test subjects. However, should such adverse events or serious adverse events occur, they will be recorded during visits. In the event of urgent AEs, subjects contact the study centre immediately, if necessary by telephone.

6. Cancellation criteria

6.1. Dropout of the individual

Each study participant has the right to withdraw their consent to participate in the study at any time without giving reasons. The study will also be cancelled for the individual if exclusion criteria 1b, c, d, 2, 3, and 5 occur. Any pregnancy occurring during participation must be reported immediately to the investigators. As soon as a pregnancy is confirmed, the subject is withdrawn. The study

intervention is not expected to have any negative effects on pregnancy (smoking cessation in particular should have a positive effect).

6.2. Cancellation of the overall study

The overall study can be terminated prematurely if

- the recruitment rate is insufficient and this deficiency appears irreparable;
- the necessary logistics can no longer be organised.

The planned study does not represent a health risk for test subjects, so that a cancellation of the study for this reason is not to be expected.
The final decision to discontinue the study lies with the principal investigator.

7. Statistical analysis

7.1. Evaluation strategy and sample size

7.1.1. Primary end criterion

The primary outcome criterion is evidence of a reduction in cancer risk for at least one of four of the female-specific cancers, defined as an absolute reduction (difference baseline-follow-up) in the WID index score in cervical smear DNA methylation signature. These scores are determined as follows: microarray analysis (Illumina EPIC array) of DNA methylation in cervical smears is performed. A score (WID-BC, WID-OC, WID-EC, WID-CIN) is then determined from the β (beta) methylation values using the four WID algorithms (linear models). To measure the primary target criterion, WID indices are tested before and after intervention (6 months) using a paired Wilcoxon test and corrected for multiple testing using the Benjamini-Hochberg correction.

7.1.2. Secondary end criteria

For further longitudinal analyses of the WID indices (multiple analyses over 0, 2, 4, 6, 12, 18, 24 and 30 months), linear mixed models are used, which allow adjustment for individual factors ("subject variables", e.g. different baseline values) and repeated measurements and possible confounding factors (e.g. adherence to the intervention by actual cigarettes smoked, actual BMI reduction, ketosis, step count, etc.). The analysis is carried out separately for the smoking cessation intervention and the diet arm. In the diet arm, the two slightly different interventions (intermittent fasting with or without ketogenic supplementation) will be compared in terms of WID score reduction.
For statistical evaluation of the other secondary outcome criteria, Wilcoxon tests or linear mixed models are also mainly used to analyse the effects of the interventions on various outcome criteria.

However, an important difference to the primary outcome criterion is that the secondary outcome criteria are exploratory in nature; depending on the distribution of the data and the nature of the various questions, the following statistical methods can be used: linear mixed models, linear/logistic regression, Wilcoxon or Student's t test, ANOVA, g-methods, etc. Secondary end criteria with regard to DNA methylation and mutation will be analysed at the EUTOPS Institute, further secondary end criteria by different research groups of the University of Innsbruck, the Medical University of Innsbruck and the UMIT. Appropriate strategies for correction for multiple testing are applied, e.g. Benjamini-Hochberg or Bonferroni correction.
The questions here are whether and to what extent the interventions lead to the following target criteria:

- Changes in DNA methylation with regard to WID index scores, age-associated signatures and genome-wide methylation in cervical smears before, during and after the intervention (0, 2, 4, 6, 12, 18, 24 and 30 months) to capture longitudinal effects and the temporal dynamics of any changes in DNA methylation
- Changes in DNA methylation in blood, oral mucosa and possibly menstrual blood (the latter only optional in premenopausal menstruating women) with regard to age-associated DNA methylation signatures and genome-wide methylation before, during and after the intervention (0, 2, 4, 6, 12, 18, 24 and 30 months)
- Absolute change in DNA mutation load in cervical and oral mucosal swabs, blood, and menstrual blood (genome-wide)
- Change in clonal haematopoiesis of indeterminate potential (CHIP), measured via mutation analysis of DNMT3A, ASXL1, JAK2 and TET2 before and after the intervention (0, 6, 12, 18, 24 and 30 months)
- Change in clinical factors before and after the intervention (0, 6, 12, 18, 24 and 30 months):
    - Smoking cessation intervention: Smoking status
    - Nutritional intervention: BMI, body composition
    - Sports medicine parameters
- Absolute or relative changes in various blood values, including:
    - Lipids, HbA1c, metabolic profile
    - Cellular measurements: distribution of different immune cell populations (% and absolute values) in peripheral blood mononuclear cells (PBMCs); activation levels of monocytes and T cells; expression of adhesion molecules
    - Inflammatory cytokines and factors and blood (plasma), including IL-1, HMGB-1, RAGE, sASC, IL-6, etc.
- Changes in the vaginal, faecal and oral microbiome

- Change in the metabolic profile in urine, faeces and saliva
- Changes in epigenetic and functional characteristics of extrinsic skin ageing and skin barrier, their reversion by lifestyle changes and a comparison of both methods of determining the biological age of the skin
- Change in stem cell fitness of endometrial stem cells from menstrual blood with regard to proliferation and pluripotency (0, 6, 12, 18, 24 and 30 months)
- Change in vascular health factors (pulse wave velocity, intima-media thickness, plaque score)
- Absolute change in the abdominal fat composition (visceral and subcutaneous)
- Change in EQ-5D-5L scores with regard to health status and quality of life before and after the intervention (6, 24 and 30 months)
- Change in psychological scale values
- Analysing psychological factors that determine compliance and effectiveness of the intervention with regard to lifestyle changes
- Reprogramming of peripheral blood cells and investigation of epigenetic reprogramming signatures after the intervention

In addition, the factors influencing compliance with the respective interventions are exploratively analysed.

As a sensitivity analysis, an adjustment for non-compliance with the intervention is carried out using causal inference methods (g-methods).

7.1.3. Case number justification

The justified case number of n=60 per study intervention is based on an initial estimate of the clinically relevant expected effect and variability: a total of 60 participants are required to detect a difference of 0.2 in the DNAme indices (e.g. smoking index), assuming a standard deviation of 0.44 (based on data from the FORECEE validation set), a statistical power of 94% and a significance level of 5%. There is no preliminary data on the longitudinal reduction of WID scores yet, therefore the TirolGESUND study also serves as a pilot study to assess effect sizes for future larger studies: for example, there is no preliminary data on differences between intermittent fasting and intermittent fasting with ketogenic supplementation with regard to DNA methylation. Thus, the TirolGESUND study will inform further future studies.

7.1.4. Expected drop-out level

In order to minimise drop-outs, we have minimised the burden on participants (as few study visits as possible by combining appointments) and will remain in regular contact with test subjects through

coaches in order to maintain compliance (even in the event of setbacks) and to underline the appreciation of each subject. The study and expectations will be fully explained to subjects at the beginning of the study so that expectations are clear. We expect a drop-out rate of 15-20% (30 out of 180 subjects), with drop-outs evenly distributed across the interventions. If subjects drop out within the first three months of the study, we will recruit one new subject for each dropped-out subject.

7.2. Randomisation

For the "diet" arm of the study, subjects will be randomised 1:1 between intermittent fasting and intermittent fasting with ketogenic supplementation. The randomisation method used is age- and BMI-stratified block randomisation in groups of 4 subjects each.

7.3. Significance level

An (adjusted) p-value < 0.05 is accepted as the general significance level for changes in the WID index score. For linear mixed models, the p-value as a significance level assessment is controversial, as it is difficult to estimate the degrees of freedom. However, p-values for significant model factors can be calculated using likelihood ratio tests or Kenward-Roger approximations.

7.4. Treatment of missing values and drop-outs

For missing values, the nature and pattern of these values will be examined and, where appropriate, data will be imputed to minimise bias through selective exclusion. The exact method of data imputation will depend on the type and pattern of missing data. To check whether data are missing completely at random (MCAR), we will apply Little's test. It is to be expected that data are not MCAR, for example due to increased drop-out or missing values due to non-compliance with the intervention. We will use multiple imputation methods for data imputation, for example MICE or Markov Monte Carlo Chain algorithms or causal inference methods [107], respectively. The respective imputation model will be checked for goodness of fit (by posterior predictive checking, PPC).

8. Data management

8.1. Data recording

Clinical data is recorded and maintained via the Askimed platform (clinical factors: baseline health check; bioelectrical impedance; vascular health and lipid measurements) or for the follow-up visits via subsequently developed data entry masks, which can only be accessed by authorised persons and also only allow authorised persons to view the entered data (clinical factors, bioelectrical

impedance and pulse wave measurement are transmitted externally), and via a secure data repository ("data repository" for laboratory values and exploratory purposes: DNA methylation, metabolome and microbiome evaluations). Measurements recorded by the study are entered by the individual collaborators or, where appropriate, by participants themselves (for IPAQ questionnaires, EQ-5D-5L), into the Askimed platform or subsequently developed data entry screens, which can only be accessed by authorised persons and only allow authorised persons to view the entered data. An electronic Case Report Form (eCRF) is maintained for each respondent in Askimed and subsequently developed data entry masks. eCRFs are pseudonymised and do not contain names, dates of birth or other personal data. Data collected by the mobile devices (fitness tracker and FORA® 6 Connect) is stored in the cloud via the respective consumer apps and transferred to our data storage and analysed using a secure API key.

8.2. Data protection concept

As part of the study, personal data (name, date of birth) and data on the intervention, health status, and biological samples (cervical smear, blood sample, cheek swab, skin sample, urine, faeces, saliva; menstrual blood; skin biopsy; see Table *3*) were collected. As part of data management and in the study database, this data is stored electronically in pseudonymised form, i.e. without direct reference to the patient's name, using a study identification number (ID) - archived in the case of biological samples - and analysed. Communication between the partners involved takes place exclusively via the ID. Only the regular blood count with regard to HbA1c and CRP, which is carried out in the laboratory of the hospital of Hall in Tirol, will appear in the electronic health record of the test subjects; test subjects will be informed of this in advance. Subjects (or their GPs) can also be informed about the results of the duplex sonography, ergometry and general health check at the sports medicine institute ISAG on request.

With the help of a security concept, protection against unauthorised access and protection against data loss is ensured in the Askimed database platform and the subsequently developed data entry masks, which can only be accessed by authorised persons and only allow authorised persons to view the entered data, and the TirolGESUND data storage system, and care is taken to ensure that the provisions of the Data Protection Act are complied with. The study data is protected from unauthorised access and only employees of the study are permitted to access it. These employees are bound to secrecy.

In the event of revocation of the declaration of consent, data will be deleted immediately, unless the processing of data already collected is authorised by the respective test person.

Study data is treated with the utmost confidentiality and access is only granted to authorised persons who need access to this data as part of the study. Due to legal regulations to ensure data quality and to monitor the conduct of the study, the cooperation partners are obliged to grant authorised third parties access to the files of the participants (source data). These include monitors, auditors and other authorised representatives of the client or employees of the responsible supervisory authority. These persons are obliged to maintain confidentiality.

Biological samples in this study are also identified only by the study identification number. The collected materials are measured (see Table 2) and stored protected from access. Only authorised personnel according to a pre-established delegation log within the framework of research and development will have access to the materials.

8.3. Quality control

Supervision and quality control of the interventions and examinations carried out in the collaborating clinics is carried out by the study management. Unclear data collection, as a large number of collaborators are involved, was identified as a potential risk for critical processes. This risk was assessed and we have implemented the following steps to minimise risks: Coordination via the study centre; central data repository for storage; compliance with pseudonymisation and tube barcoding / LIMS automation.

9. Legal and ethical aspects

9.1. Declaration of Helsinki

The study is conducted in accordance with the current version of the Declaration of Helsinki.

9.2. Ethics Committee

The study protocol will be submitted to the Ethics Committee of the Medical University of Innsbruck for review before the start of the study.

9.3. Good clinical practice (ICH-GCP)

The study is conducted in accordance with ICH-GHP (E6).

9.4. Local and national regulations

The TirolGESUND study is conducted in accordance with the regulations of local and national authorities.

9.5. Data protection

The names of the test subjects and all other confidential information are subject to medical confidentiality, the provisions of the Data Protection Act Tyrol and the Federal Data Protection Act, and the GDPR (General Data Protection Regulation).

9.6. Voluntary participation and cancellation

Participation in the TirolGESUND study is voluntary. Consent can be given by test subjects can be withdrawn at any time, without giving reasons and without disadvantages for further medical care. The study participants will be informed verbally and in writing about the nature and scope of the planned study before the start of the study. Their consent to participate is documented by signing the informed consent form. If a participant withdraws from the study, they will be asked whether they agree to the material being analysed and archived. If not, the (data) material already obtained will be destroyed.

9.7. Incentives for test subjects

Participation in the TirolGESUND study is unpaid, but the test subjects receive free counselling in the form of a professional smoking cessation programme, dietary advice and exercise protocols, and 1:1 psychological coaching to adopt and maintain a healthy lifestyle. This motivation, in conjunction with participation in a scientific study, is seen as an incentive for participants.

9.8. Financing

The study is funded by the principal investigator (EUTOPS Institute budget and H2020 HEAP Grant) and the sponsor. Some costs are covered by (scientific) cooperation partners as in-kind contributions.

9.9. Changes to the clinical trial protocol

Significant changes to the protocol are not foreseen. Should these occur (e.g. change of intervention or primary endpoints), they will be reported to the ethics committee.

9.10. Retention obligation

In accordance with the Tyrolean Hospital and Sanatorium Act, data (age, gender, biological samples, etc.) must be stored for at least 10 years. For future research projects, samples and data will be stored in the International Biobank Zams (Hauptstr. 100, 6511 Zams) for up to 20 years after the end of the study. Biological samples and associated epidemiological data are only stored without directly personal data and only authorised employees of potential future research projects in the context of disease and cancer prevention have access to them. Samples and data will be destroyed at the request of the subject. Data and/or biomaterials are not commercially utilised.

9.11. Test plan deviations

Compliance with the intervention is regularly monitored by coaches. However, if test subjects do not adhere to the interventions, this does not lead to exclusion from the study, as this information can also be valuable. Studies that record the effects of lifestyle interventions on biological parameters have not yet been conducted on this scale. This study is therefore also a pilot study to determine how compliant female subjects are in such comprehensive studies and what drop-out rates can be expected in any future studies. In addition, the parameters of women who do not adhere to the intervention can also be useful and can be used as an indirect "nested control" (i.e. "what happens without intervention?"). For ethical and practical reasons, it is almost impossible to recruit a control group without intervention and thus determine that subjects "must" smoke for at least another 6 months).

In order to extract as much information as possible, compliance is regularly checked by coaches. In the case of non-compliance, we will distinguish between one-off deviations from the protocol (for example: smoking a few cigarettes on one occasion; intermittent fasting not adhered to for a few days) and long-term non-compliance (continuous non-adherence to the intervention for two weeks or more).

9.12. Insurance

The study insurance was taken out with Zürich Versicherungs-Aktiengesellschaft (Schwarzenbergplatz 15, A-1010 Vienna, Tel. 08000 80 80 80) under the policy number 07208763-1.

9.13. Conflicts of interest

The principal investigator is a partner in the companies SOLA Diagnostics GesmbH (development of methods to determine the risk of cancer) and Preediacan (development of methods for the early detection of cancer). The TirolGESUND study is partly funded by the EUTOPS (funded by the

province of Tyrol) and the H2020 HEAP Grant (funded by the EU), whose director or Principle Investigator is the principal investigator of the TirolGESUND study (Univ.-Prof. Dr Martin Widschwendter).

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11. Appendix

A. SOP: Smoking cessation intervention

Smoking cessation according to IFT (https://www.rauchfrei-programm.de)

The smoke-free programme combines the latest concepts of motivational research and behavioural therapy. The methods used have proven to be effective in clinical studies and meta-analyses and therefore comply with the guidelines of the scientific societies (AWMF guidelines) and recommendations of the World Health Organisation (WHO Europe) for the treatment of tobacco addiction.

The smoke-free programme enables comprehensive and lasting smoking cessation with the core contents "Observe, Change and Stabilise".

In the small group, the course participants - supported by their active participation and with the help of the accompanying book - learn in the course of 3 appointments and two individual telephone consultations to better understand their smoking behaviour, to question the motivation that causes them to smoke and to develop coping strategies.

Participants are comprehensively prepared by the trainers (certified addiction counsellors, psychologists, psychotherapists) for a fixed stop-smoking day (after the third course unit) and are then supported and guided in remaining smoke-free.

In addition, an exercise unit takes place as part of the smoke-free programme. The content of the movement unit is related to the theoretical part of the smoking seminar. In this movement unit, light exercises on the subject of breathing, relaxation and rhythm are practised.

Smoking cessation

- 3 appointments of 180 min each (= 2 course units) every week
- 1 appointment for exercise of 60 min
- Group setting (from 6 participants - 12 participants)
- Two individual telephone counselling sessions per participant

Characteristics of the programme

- Up-to-date - the training programme is characterised by a compact, didactically structured approach
- Transparent - a standardised, detailed manual is available
- Targeted - the focus is on the positive aspects of a smoke-free life
- Sustainable - the focus is on long-term stabilisation of initial successes
- Responsible - no unrealistic promises are made
- Successful - the most scientifically successful procedures are combined
- Quality-assured - the course concept includes a standard evaluation

B. SOP: Smoking cessation intervention - accompanying dietary measures

Tirol GESUND - Smoking cessation

Accompanying dietological measures and monitoring of nutritional status

As part of the pilot study on smoking cessation, the accompanying dietological care and monitoring of the nutritional status of the test subjects will take place.

Body length (KL) and body weight (KG) are measured to assess the nutritional status. KL is recorded at the beginning (T0) and KG at the beginning and after 2 (T2), 4 (T4) and 6 (T6) months. Dietary logs are kept in the week before the study visits (T0, T2, T4, T6) to give the dieticians an insight into the subjects' eating behaviour. In addition, at the beginning, after 2, 4 and 6 months, the subjects received nutritional counselling regarding the implementation of a balanced mixed diet. Figure 1 shows the accompanying dietological support and the monitoring of the nutritional status of all test subjects in the Tirol GESUND smoking cessation study arm.

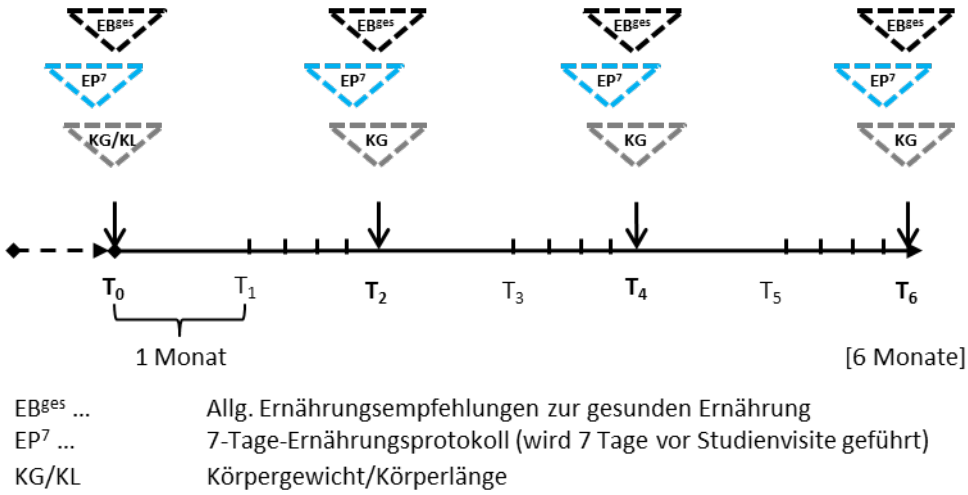


Fig. 1: Accompanying dietological care and monitoring of nutritional status in the smoking cessation study arm.

C. SOP: Intervention Intermittent fasting

Tirol GESUND - Weight loss

Intermittent fasting and monitoring of nutritional status

As part of the pilot study on weight loss, a nutritional therapeutic intervention in the form of intermittent fasting (IF) and monitoring of the nutritional status of the test subjects will be carried out.

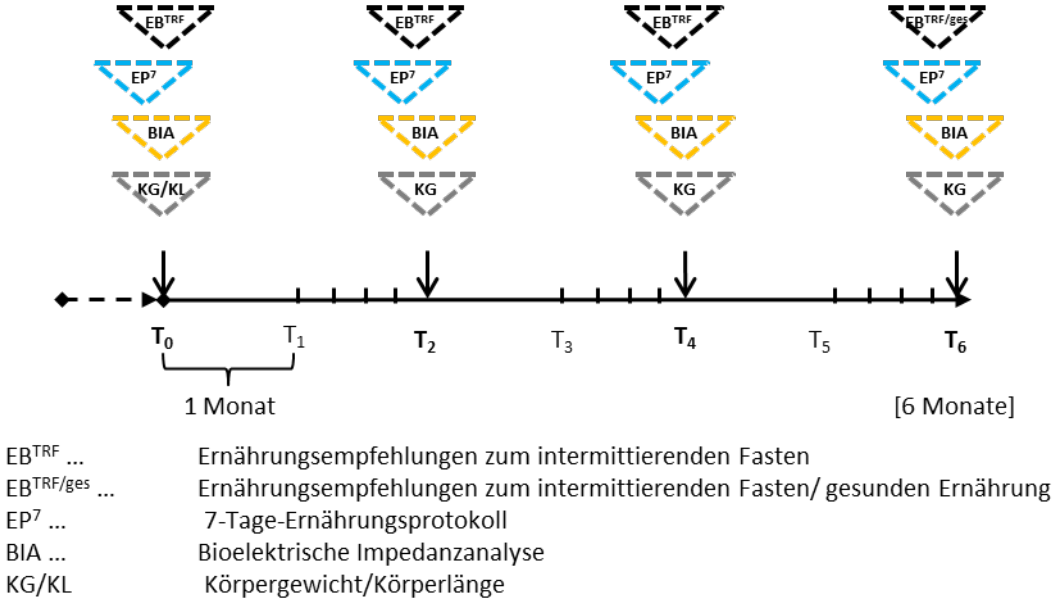
Body length (KL) and body weight (KG) are measured to assess the nutritional status. KL is measured at the beginning (T0) and KG at the beginning and after 2 (T2), 4 (T4) and 6 (T6) months. Body composition measurements (bioelectrical impedance analysis) and the evaluation of dietary protocols are also carried out at the beginning, after 2, 4 and 6 months. Nutrition logs are kept in the week before the study visits (T0, T2, T4, T6). In addition, the β-hydroxybutyrate values in the capillary blood are measured three times a week on the fasting days to assess the metabolic change using the FORA 6 Connect device, which is made available to test subjects.

Based on the work published by de Cabo and Mattson (de Cabo and Mattson 2019: N Engl J Med 2019; 381:2541-2551), the shortened stepwise introduction of a "Time Restricted Feeding" (TRF) regime is carried out with T0 in the following way:

- Week 1: 10 hours of food intake, 14 hours off food for 5 days per week
- Week 2: 8 hours of food intake, 16 hours off food for 5 days per week
- Week 3 and subsequent weeks: 8 hours of food intake, 16 hours off food for 7 days per week
- *Week 4 and subsequent weeks: 6 hours of food intake, 18 hours of abstinence from food for 7 days per week (optional)*

In addition, one of the groups will be given a ketogenic diet to reinforce ketosis for the entire duration of the study. This is done once a day before the start of the fasting phase. After the induction phase, which extends over the first month, a daily fasting interval of up to 16 hours is to be maintained for the remaining study duration of approx. 5 months. An optional extension to an 18-hour fasting phase is possible. The timing of the fasting phases is left to the participants themselves. As part of the nutritional counselling at the start of the study, the subjects will be advised on the practical implementation of the IF regime based on their previous diet (according to the EP). Exemplary daily plans for achieving the specified fasting phases are discussed. The counselling sessions after 2 and 4 months serve to

monitor the dietary progress and motivate the subjects to continue the TRF regime. After 6 months, counselling is provided regarding the gradual termination of the TRF regime in reverse order to the introduction and continuation of a balanced mixed diet or, in the case of an optional extension of the study, the ongoing dietary support of the TRF regime.
Figure 2 shows the dietary support of the TRF regime and the monitoring of the nutritional status of all subjects in the weight reduction arm of the Tirol GESUND study.



EB^{TRF} ...	Ernährungsempfehlungen zum intermittierenden Fasten
$EB^{TRF/ges}$ ...	Ernährungsempfehlungen zum intermittierenden Fasten/ gesunden Ernährung
EP^{7} ...	7-Tage-Ernährungsprotokoll
BIA ...	Bioelektrische Impedanzanalyse
KG/KL	Körpergewicht/Körperlänge

Fig. 2: IF regime and monitoring of the nutritional status of all subjects in the weight loss arm of the study

D. Nutrition protocol

Name:
Date of birth:
*Serial no. No.**
*Week/day of fasting**

**will be filled in by the study team*

Day of the week (date):

Time of day	Food	Drinks	Remarks

E. IPAQ questionnaire

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

SELF-COMPLETION LONG VERSION FOR THE LAST 7 DAYS

FOR USE BY YOUNG PEOPLE AND ADULTS OF MIDDLE AGE (15-69 years)

The International Physical Activity Questionnaire (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains surveyed independently) and short (4 general items) versions are available for both telephone and self-administered methods. The intention of the questionaires is to provide simple instruments that can be used to obtain internationally comparable data on health-promoting physical activity.

Background of the IPAQ

The development of an international physical activity measurement instrument began in Geneva in 1998 and continued in 2000 through extensive reliability and validity testing in 12 different countries (14 sites). The final result is claimed to have acceptable measurement properties for use in many locations and in different languages and to be suitable for nationwide population-based studies of the prevalence of participation in physical activity.

Using the IPAQ

It is recommended to use the IPAQ instruments for examinations and research purposes. The arrangement of the questions and the sentence positions should remain as unchanged as possible so as not to influence the psychometric properties of the instrument.

Translation from English and cultural adaptation

Translations from English are being sought to facilitate the worldwide use of the IPAQ. Information on the availability of the IPAQ in different languages can be found at www.ipaq.ki.se. Should a new translation be undertaken, it is strongly recommended that the back-translation methods described on the IPAQ website are used. If possible, please consider making your translation of the IPAQ available to others on the IPAQ website. Further details on translations and cultural adaptations can be downloaded from the website.

Further developments of the IPAQ

International co-operation on the IPAQ continues and the ***International Physical Activity*** Study is in the development phase. Further information is available on the IPAQ website.

Further information

Detailed information on research methods used in the development of the IPAQ instruments can be found at www.ipaq.ki.se or at Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Further scientific publications and presentations on the application of the IPAQ are summarised on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out what types of physical activity people do in their daily lives. The survey refers to the time you have spent in physical activity during the **last 7** days. Please answer all questions (even if you do not consider yourself an active person). Please take into account the activities you do at work, in your house and garden, to get from one place to another and in your free time for recreation, exercise and sport.

Think about all your **strenuous** and **moderate** activities in the **past 7 days**. **Strenuous** activities are activities that require a lot of physical effort and during which you breathe much more heavily than normal. **Moderate activities refer to activities** with moderate physical exertion during which you breathe a little harder than normal.

A. PART 1: PHYSICAL ACTIVITY AT THE WORKPLACE

The first section is about your work. This includes paid work, farming, voluntary work, seminars and any other unpaid work you have done outside the home. Do not include any unpaid work you have done at home, such as house and garden work, maintenance work and caring for the family. This will be asked in section 3.

1. do you currently have a job or do you do any unpaid work outside the home?

☐ Yes

☐ No → ***Skip to part 2: PROMOTION***

The following questions are about physical activity in the **past 7** days as part of your paid and unpaid work. This does not include travelling to or from work.

2. on how many of the **past 7 days** have you performed strenuous physical activities such as heavy lifting, digging, heavy construction work or climbing stairs as **part of your job**? Think only of physical activities that you performed for at least 10 minutes without interruption.

_____ **Days per week**

☐ No strenuous physical activities as part of work

→ **Skip to question 4**

3. how much time did you usually spend on one of these days doing **strenuous** physical activity as part of your work?

_____ **Hours per day**
_____ **Minutes per day**

4 Again, think only of the physical activities that you did for at least 10 minutes without interruption. On how many of the **past 7 days** have you performed moderate physical activities such as carrying light loads as **part of your work**? Please do not include walking.

_____ **Days per week**

☐ No moderate physical activity at work

→ **Skip to question 6**

5. how much time did you usually spend on one of these days doing moderate physical activity as part of your work?

_____ **Hours per day**
_____ **Minutes per day**

6. on how many of the **past 7 days** have you **walked distances** of at least 10 minutes without interruption as part of your work? Please do not include journeys to or from work.

_____ **Days per week**

☐ No walking routes as part of the work

→ ***Skip to part 2: PROMOTION***

7. how much time did you usually spend **travelling as** part of your work on one of these days?

_____ **Hours per day**
_____ **Minutes per day**

part 2: physical activity for promotion

These questions are about getting from one place to another, such as going to work, shops, the cinema, etc.

8. on how many of the **past 7 days** have you **travelled by motorised transport** such as train, bus, car or tram?

_____ **Days per week**

☐ No journeys in motorised means of transport

→ ***Skip to question 10***

9. how much time did you usually spend **travelling** by train, bus, car, tram or any motorised transport on any of these days?

_____ **Hours per day**
_____ **Minutes per day**

Just think of **cycling** and **walking,** where you have travelled to and from work, for errands and for journeys from one place to another.

10. on how many of the **past 7 days** have you **cycled** for at least 10 minutes without stopping to get **from one place to another?**

_____ **Days per week**

☐ No cycling from one place to another

→ ***Skip to question 12***

11. how much time did you usually spend **cycling from** one place to another on one of these days?

_____ **Hours per day**
_____ **Minutes per day**

12. on how many of the **last 7 days did** you **walk** for at least 10 minutes without stopping to get **from one place to another**?

_____ **Days per week**

☐ No walking from one place to another

→ ***Skip to Part 3: HOUSEWORK, HOME MAINTENANCE AND FAMILY CARE***

13. how much time did you usually spend **walking** from one place to another on one of these days?

_____ **Hours per day**
_____ **Minutes per day**

PART 3: HOUSEWORK, HOME MAINTENANCE AND CARING FOR THE FAMILY

This section is about physical activities you have done in and around your home in the **past 7 days**, such as housework, yard and garden work, maintenance work and caring for the family.

14 Think only of the physical activities you have done for at least 10 minutes without interruption. In how many of the **past 7 days have you performed** strenuous physical activities such as carrying heavy loads, picking up wood, shovelling snow or digging **in the yard or garden**?

_____ **Days per week**

☐ No strenuous physical activity in the yard or garden

→ ***Skip to question 16***

15. how much time did you usually spend on one of these days with **strenuous** activity in the garden and yard?

_____ **Hours per day**
_____ **Minutes per day**

16 Again, think only of the physical activities that you have done for at least 10 minutes without interruption. On how many of the **past 7 days** have you performed moderate activities such as carrying light loads, sweeping, window cleaning and raking **in the yard or garden**?

_____ **Days per week**

☐ No moderate activity in the garden or yard

→ ***Skip to question 18***

17. on one of these days, how much time did you usually spend in the garden or yard doing **moderate** physical activity?

_____ **Hours per day**
_____ **Minutes per day**

18 Again, think only of the physical activities that you have done for at least 10 minutes without interruption. On how many of the **past 7 days have you performed** moderate activities such as carrying light loads, cleaning windows, washing floors and sweeping **at home**?

_____ **Days per week**

☐ No moderate activities at home

→ ***Skip to part 4: PHYSICAL ACTIVITIES IN RECREATION, SPORT AND LEISURE***

19. how much time did you usually spend doing **moderate** physical activity at home on one of these days?

_____ **Hours per day**
_____ **Minutes per day**

PART 4: PHYSICAL ACTIVITIES IN RECREATION; SPORT AND LEISURE

This section is about all physical activities you have done in the **past 7 days,** exclusively in recreation, sports, physical exercises and leisure time. Please do not include any activities that you have already listed.

20. excluding the walks you have already mentioned, on how many of the **last 7 days did you walk** for at least 10 minutes without stopping in your **free time**?

_____ **Days per week**

☐ No walking in your free time

→ ***Skip to question 22***

21. how much time did you usually spend walking in your free time on one of these days?

_____ **Hours per day**
_____ **Minutes per day**

22 Think only of the physical activities you have done for at least 10 minutes without interruption. How many of the **past 7 days** have you done **strenuous** physical activity such as aerobics, running, fast cycling or fast swimming in your **free time**?

_____ **Days per week**

☐ No strenuous activities in your free time

→ ***Skip to question 24***

23. how much time did you usually spend on one of these days doing **strenuous** physical activity in your free time?

_____ **Hours per day**
_____ **Minutes per day**

24 Again, think only of the physical activities you have done for at least 10 minutes without interruption. On how many of the **past 7 days** have you done **moderate** physical activities such as cycling at a normal speed, swimming at a normal speed and doubles tennis in your **free time**?

_____ **Days per week**

☐ No moderate activities in leisure time

→ ***Skip to part 5: TIME SPENT SITTING***

25. how much time did you usually spend doing **moderate** physical activity in your free time on one of these days?

_____ **Hours per day**
_____ **Minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spent sitting at work, at home, at seminars and in your free time. This can include time sitting at a desk, visiting friends and sitting or lying in front of the TV. Do not include any time spent sitting in motorised transport that you have already told me about.

26. how much time have you spent **sitting** on **weekdays in the past 7 days**?

_____ **Hours per day**
_____ **Minutes per day**

27.in the **past 7 days** , how much time did you spend **sitting** on **weekend days**?

_____ **Hours per day**
_____ **Minutes per tag**

F. SOP: Saliva samples for metabolic profiling

Samples:

Native saliva samples

Collection and transport of samples:

- Subjects are instructed not to eat, drink, smoke or use oral hygiene products for at least one hour prior to sample collection. Therefore, saliva samples are best collected in the morning before brushing teeth.
- The mouth should be rinsed with tap water
- Approximately 5 minutes later, saliva is transferred by spitting into the sterile container provided. Ideally, 2 ml (at least 1 ml) of saliva should be collected.
- Close the container.
- As a basic rule, the time between collecting the saliva and providing the sample should be as short as possible. The sample must be stored in a cool place (please do not freeze): at 4°C or on ice or between cool packs fresh from the freezer / freezer compartment, replace ice or cool packs if necessary. The samples will keep for a maximum of 8 hours.
- Transport to the laboratory also on ice or embedded between cool packs

Processing of the samples after delivery to the laboratory:

After the samples have been delivered, they must be centrifuged (2000 x *g*, 10 min, 4°C). The supernatant is transferred to fresh containers and stored at -80°C (longer-term storage possible).

Sample quantity required for the analyses at least 1 mL (a total quantity of at least 2 mL is desirable for the preparation of additional quality controls).

G. SOP: Metabolic profiling of urine samples

universität innsbruck

Institut für Pharmazie

Organisational unit	Manager	Date: 25.05.2020
Pharmacognosy	University Professor Dr Hermann Stuppner	

Metabolic profiling of urine samples

Samples:

Smoking cessation intervention: First morning urine (midstream urine)

Should be collected before breakfast, on an empty stomach.

Samples are not collected during menstruation.

Nutritional intervention: Last urine before food intake (midstream urine)

Collecting and transporting the samples

Instructions for study participants:

- Wash your hands.
- Spread labia
- Clean the genital area (vulva and urethral opening) with sterile swabs and tap water, without soap. Only open the collection container immediately before use, without touching it.
- Let the first small portion of urine flow into the toilet
- Collect the middle portion of urine in the collection container (fill to halfway). The collection vessel should not touch the body.
- Allow the remaining urine to flow into the toilet
- Close the container.
- As a basic rule, the time between collecting the urine and providing the sample should be as short as possible. The sample must be stored in a cool place (please do not freeze): at 4°C or on ice or between cool packs fresh from the freezer / freezer compartment, replace ice or cool packs if necessary. The samples can be kept for a maximum of 8 hours
- Transport to the laboratory also on ice or embedded between cool packs

Processing of the samples after delivery to the laboratory:

After the samples have been delivered, they must be centrifuged (e.g. 2000 x *g*, 10 min, 4°C). The supernatant is transferred to fresh containers and stored at -80°C (longer-term storage possible).

Sample quantity required for the analyses at least 1 mL (a total quantity of at least 2 mL is desirable for the preparation of additional quality controls).

Institute of Pharmacy/Pharmacognosy , Innrain 80-82, CCB-Centre for Chemistry and Biomedicine, 6020 Innsbruck , Austria

SOP: Biobank IBD - Sample collection

Author: Simon Reider, Alexander Moschen

Version: v1, 22.5.2020

1. Fecal samples

a) Native fecal sample:

- Collection: Sarstedt® Fecal collection Tubes (e.g. 80.734.301)
- Amount: approximately min. 5g feces
- Transport: store at 4°C if possible between collection and study visit. Transport at ambient temperature
- Aliquots: n = 3 á 100 mg
- Storage: immediately at -80°C
- Analytic targets: fecal metabolome

b) Stabilised fecal sample:

- Collection: Zymo DNA/RNA Shield fecal collection tube (Zymo # R1101)
- Amount: according to manufacturer's protocol (use provided spoon)
- Transport: can be kept at room temperature for 4 weeks
- Aliquots: n = 3
- Storage: at -20 or -80 °C
- Analytic targets: fecal microbiome (16S and/or shotgun sequencing), fecal metatranscriptome

2. Blood Samples

- Collection: Sarstedt 10 mL Monovettes (Serum and EDTA)
- Amount: 10 mL serum and 10 mL EDTA monovette
- Transport: 4°C
- Aliquots: n = 3 á 700 µL
- Storage: at -80 °C
- Analytic targets: Cytokines

3. Saliva Samples

- Collection: DNA/RNA Shield Saliva Collection Kit (Zymo R1210)
- Amount: 2 mL
- Transport: can be kept at room temperature for 4 weeks
- Aliquots: n = 2
- Storage: at -20 or -80 °C
- Analytic targets: oral microbiome (16S and/or shotgun sequencing), oral metatranscriptome

Saliva sample collection:

Subjects are instructed not to eat, drink, smoke or use oral hygiene products for at least one hour prior to sample collection. Therefore, saliva samples are best collected in the morning before brushing the teeth. The mouth should be rinsed with tap water.
Saliva samples for metagenome/metatranscriptome are collected in tubes with stabilising solution according to the manufacturer's instructions (Zymo Research DNA/RNA Shield Saliva Collection Kit R1210). For this purpose, 2 mL of saliva is collected from the test person using a funnel in a sample tube; a corresponding calibration mark is provided. The stabilising solution (DNA/RNA Shield) contained in the kit is then added via the funnel and the tube is sealed. Finally, the sample tube is inverted 10 times to ensure sufficient mixing of the stabiliser with the sample.
The stabilised sample can then be stored for up to 1 month at room temperature or indefinitely at -20 °C to -80 °C.

H. SOP: Bioelectrical impedance analysis

1. **Aim & purpose of the BIA measurement**
    - Assessment of body composition (fat mass, active body cell mass and free fluid)
    - Encourage motivation with regard to weight stabilisation and weight loss

2. **Timing**
    - Measurement: approx. 15', discussion: approx. 30'

3. **Materials**
    - BIA measuring device (BIA Corpus RX 4000M)
    - Lounger with crepe roll
    - Skin disinfectant, swabs (for skin cleansing)
    - BIA electrodes (BIANOSTIC AT, Data Input)
    - PC/ laptop + evaluation programme of the measurement results (Body Comp in the respective updated version)

4. **Measurement requirements**
    - Current body weight
    - Current body length
    - **The last large main meal should have been 2-3 hours ago, fluid intake: pause ½ to 1 hour before the measurement**
    - **Urinary bladder should be emptied before the measurement**
    - **Sport: no sporting activity for > 8 hours before the measurement**
    - The electrodes must be able to adhere, therefore the skin areas to which the measuring electrodes are attached must be cleaned
    - Electrodes: use special BIA measuring electrodes, store in an airtight container, disposable products

5. **Contraindications**
    - Pacemaker
    - Defibrillator
    - Life-sustaining, pulse-generating or substance-delivering implanted systems

6. **Measuring procedure**
    - see supplement

7. **Frequency of realisation**
    - Start of study, after 2, 4 and 6 months

8. **Annual maintenance of the BIA measuring device**

Measurement procedure:

- The test person should remain lying flat on a suitable patient couch between 5 - 10 minutes before the measurement (during this time the other preparations for the measurement should be carried out). The metal of the couch should not be touched.
- Arms should not rest against the upper body (distance approx. 30°), legs should not touch the inside of the thighs (distance approx. 45°), see Fig. 1.
- Skin areas should be degreased and cleaned with skin disinfectant before placing the electrodes.

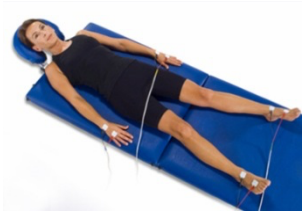


Fig. 1: Correct body position

- Correct electrode placement:
  The distance between the electrodes should be 5 cm (approx. 3 fingers wide).

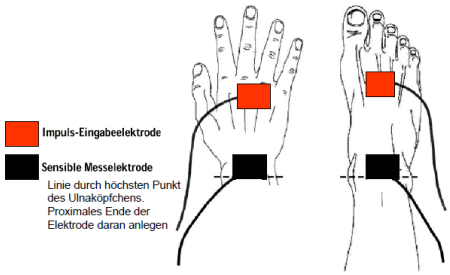


Fig. 2: Electrode placement

- Measuring cables must be connected to the electrodes in accordance with the device instructions (see Fig. 3).
- The measuring device should only be switched on at the start of the measurement.

Fig. 3: BIA device currently in use

- Measurement data (Rz, Xc) are entered in the form provided or directly via Body Comp and are then available for results analysis and interpretation.

Espen Guidelines (Bioelectrical impedance analysis - part II: utilisation in clinical practice), Clinical Nutrition (2004) 23, 1430-1453
Images from: Guide to BIA evaluation Implementation and interpretation of phase-sensitive BIA measurements with BodyComp V 8.5. AENGUS Ernährungskonzepte GmbH 2011.

I. Bioelectrical impedance measurement protocol

Bioelectrical impedance analysis measurement

I. Test person data

Number:		
Name:		Note:
Date of birth:		
Age:	years	
Body weight:	kg	
Body size:	m	
BMI:	kg/m²	

II. . Measurement, on

	RARF	RALA	RFLF	LALF
RZ				
XC				

III. Comment:

☐ A. Höller
(+43 […])

☐ U. Pichler
(+43 […])

J. SOP: Vascular health measurements

- **Pulse-wave analysis and pulse-wave velocity measurement**

Pulse-wave velocity (PWV) measurements are conducted in a supine position after a resting period of at least 5 minutes with head and shoulders elevated to 30 degrees. For the PWV measurement one cuff is installed around the neck with the sensor situated over the centre part of the (right) common carotid artery which is palpated prior to the application and the other cuff is installed around the (right) upper limb (as high to the groin as possible). For the calculation of the PWV, the distance is taken between the suprasternal notch and the centre of the femoral cuff is measured diagonally (to an accuracy of 0.5 cm). In any case, where a fairly flat distance reading is not possible (e.g. obese subject), the distance device (caliper) is used for distance measurement.

For the pulse-wave analysis, the cuff is placed around the upper arm (as high as possible). Simultaneous oscillometric recording of 10 consecutive heart cycles without artefacts allows for the detection of central blood pressure, augmentation index and PWV by means of the pulse-wave shape and duration using the Vicorder (SMT medical, Würzburg, Germany).

Pulse-waves are checked for their quality and plausibility of the results has to be questioned.

- **Cervical Vessel Artery Ultrasound**

Duplex sonography of the internal, external and common carotid as well as both vertebral arteries is conducted in a supine position by a standard vascular ultrasound machine. Maximum intima-media-thickness, maximum plaque size and vessel diameter is measured at predefined segments of the common (1.5 to 3 cm and 0 to 1.5 cm proximal to the carotid bulb) and internal (1.5 to 3 cm and 0 to 1.5 cm distal to the carotid bulb) carotid arteries. Maximum flow and vessel diameter is measured in the V2-Segment of both vertebral arteries. Vessel stenosis are graduated according to the definitions of the European Carotid Surgery Trial (ECST).

K. SOP: Skin biopsy collection and skin barrier measurement

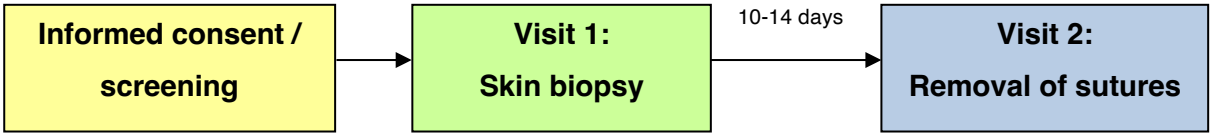
1st objective

The primary objective of this study is to collect human tissue and measure skin barrier function from healthy volunteers during the TirolGESUND trial that will be used to facilitate further research including generation of cell cultures and various subsequent laboratory analyses.
The tissue that will be collected in the form of a punch biopsy will be taken from the inner arm or leg.

1.1 Protocol: Skin Biopsies and Barrier Measurements from Healthy Volunteers

After providing informed consent, volunteers that agree to provide skin biopsies will be examined to confirm their health status and suitability for participation in the study. Once confirmed, they will have non-invasive skin barrier measurements and 2-3 biopsies of their skin removed using a 6 or 8 mm punch under local anaesthesia. The wound will be closed with sutures and volunteer released after stabilisation. The volunteer will return to the clinic or to her/his practioner 10-14 days later to ensure there were no unexpected adverse effects of the procedure and to have the sutures removed (if necessary).

Figure 1: Schematic of visits for collection of skin biopsies



2. selection and withdrawl of volunteers

The following exclusion criteria are designed to select subjects for whom a skin biopsy is considered appropriate. All relevant medical and non-medical conditions will be taken into consideration when deciding whether this protocol is suitable for a particular subject.

2.1 Exclusion criteria for skin biopsies

The presence of any of the following will exclude a subject from skin biopsies and barrier assessments:

1. Presence of any medical condition that influences the skin health (e.g. lichen planopilaris, lupus erythematosus, severe seborrheic eczema, psoriasis, untreated thyroid gland disease/goiter development, auto-immune diseases, etc.).
2. Known infection with human immunodeficiency virus (HIV), hepatitis or syphilis.
3. Chronic intake of substances affecting blood coagulation, which in the investigator's opinion would impact volunteer safety
4. Allergy or known hypersensitivity against disinfection solutions, local anaesthesia, suture material and latex
5. History of complications at wound healing and former operations

2.2 Withdrawal of volunteers

Subjects have the right to withdraw from the project at any time and in any case without giving reason. Although a subject is not obliged to give his/her reason for withdrawing, the investigator should make a reasonable effort to ascertain the reason, while fully respecting the subject's right.

The investigator has the right to withdraw a subject for any reason which is in the best interests of the subject, including intercurrent illnesses, adverse events.

Whenever a subject is withdrawn from the project, for whatever reason, a final evaluation must be completed for that subject, stating the reason for withdrawal. If a volunteer does not return for a scheduled visit, every effort should be made to contact the patient. In any circumstance, every effort should be made to document the volunteer's outcome.

The total project is discontinued if investigator cannot justify further continuing due to ethical and medical reasons, e.g. serious adverse events or adverse events in high frequency.

2.2.1 Screening visit

The Screening Visit and Visit 1 can take place on the same day or on separate 5 days prior to Visit 1.

All volunteers will participate in the Screening Visit. The following procedures will take place during the Screening Visit (in chronological order):

- Review of volunteer's eligibility for study entry (exclusion criteria etc.)
- Record volunteer's demographic information
- Review volunteer's medical history and perform physical examination

2.2.2 Visit 1 (Day 0)

The following procedures will take place for all study participants during Visit 1:

- Review of volunteer's eligibility for study entry (see exclusion criteria)
- Perform physical exam (if Visit 1 not performed on same day as Screening Visit)
- Prepare skin for biopsies
- Harvest skin biopsy and suture wound

2.2.3 Visit 2 (Day 10-14)

Visit 2 will take place 10-14 days after Visit 1 with the intention to remove the volunteers' sutures from Visit 1 (if necessary) and assess volunteer condition. The following procedures will take place during Visit 2:

- Perform brief physical exam and judgement of the wound healing process.
- Removal of biopsy sutures (if necessary)

2.3 Physical Examination

In volunteers of scenario 1, dermatologist examines the area of skin biopsy and excludes the presence of erythema, active skin disease, dark pigmentation or scars that may confound study results. Furthermore a uniform skin colour is confirmed during this examination.

The investigator will judge the clinical relevance of physical examination and has to decide if a volunteer will be suitable to participate in this project or not.

2.4 Skin Biopsy and Skin Barrier Assessment Procedure s

Skin Barrier Measurements using tape strips and non-invasive measurements of transepidermal water loss (TEWL) are taken and 2-3 6 or 8mm biopsies are to be taken from the skin of the inner arm or leg.

Before biopsy, the area will be disinfected and anaesthetised. Once the local anaesthetic has taken affect a 6 or 8mm diameter deep punch biopsy will be performed and the tissue transferred to a sample vial. The biopsy site will be closed with sutures which will be removed 10-14 days later at Visit 2. Instructions for volunteers on skin care will be provided.

2.5 Study site

Sampling of biopsies will be performed at:

- University Medical Center of Innsbruck, Department of Dermatology, Venereology and Allergy (Prof. Dr Matthias Schmuth).

2.5.1 Documentation of adverse events

At each visit, any changes in physical conditions are asked for and recorded as adverse events. Possible concomitant medication is recorded and severity and outcome is evaluated by investigator. All distinctive features (adverse events/serious adverse events defined in accordance to ICH/GCP Guidelines) are inquired and documented. Seriousness criteria are defined according to ICH-GCP.

3. risk assessment (refers to donors of skin biopsies)

Risks associated with the conduct of the procedure are very low. At all times, subjects can contact a physician in the study centre.
The sampling of skin biopsies is part of medical routine and should only imply low risks under proper performance. However, it is possible that slight bleeding, haematoma, infections, impairment of nerves, slight pain of the wound, dehiscence of the wound in tension and the emergence of a small scar occur. In very rare cases a change of pigmentation at the site of biopsy puncture can occur. In very rare cases hypersensitivity against compounds of local anesthetics could occur.
At visit 2 investigator controls wound healing, removes sutures and decides about necessary further actions.

4 Responsibilities of the investigator

4.1 Adherence to the Protocol

The investigator has to approve this protocol by signing the signature page. The investigator confirms with his/her signature that the trial will be performed in compliance with this protocol.

4.2 Record Keeping by the Investigator

The investigator will maintain all essential documents for the following periods:

- All essential documents e.g. hospital records/office charts and other source documents for the longest period possible, but at least 10 years.

If the Investigator relocates, retires, or for any reason withdraws from the study, the study records may be transferred to an acceptable designee, such as another Investigator or another institution.

5 Administrative and ethical aspects

5.1 Ethics

The sampling of human tissue will be carried out in accordance with the Declaration of Helsinki (1996) and in orientation to ICH-GCP guidelines.

5.2 Informed consent

All volunteers will be informed, both verbally and in writing, about the nature of the study, the anticipated benefits and risks, any possible discomfort to which they will be exposed, and their right to discontinue their participation at any time according to their own free will. Each volunteer will confirm his/her consent in writing, prior to inclusion.

Before written informed consent is obtained, the investigator will provide the volunteer ample time and opportunity to inquire about details of the trial and to decide whether or not to participate in the trial. The written informed consent form should be signed and personally dated by the volunteer and by the investigator. Only volunteers who have voluntarily signed the informed consent form are eligible for trial participation.

The original signed informed consent will remain in the investigator's trial file. The investigator will document in the CRF that the volunteer has given his/her written informed consent. Each volunteer will receive a copy of the written informed consent.

5.3 Volunteer Insurance

A volunteer insurance will be put in place for the healthy volunteers participating in the study who give informed consent to participate in the clinical trial.

5.4 Volunteer privacy and confidentiality

The principles of the volunteer's right to protection against invasion of privacy are confirmed. Individual subject medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited. The investigator must ensure that the subject's pseudonymity will be maintained. The study site keeps a separate confidential enrolment log which matches identifying codes with the subject's names and addresses. This list is under strict confidence by investigator. Informed consent highlights information about data confidentiality.

5.5 Archiving

After cessation of the whole project, all study records, especially originals of informed consent, enrolment list and copies of all clinical study material must be archived for a period of at least 10 years. All documents must be archived in a secure place and treated as confidential material.

Participating Study Site and Investigator Agreement Signature Page

I agree that I have carefully read and understood this protocol and confirm that I will conduct this clinical study in accordance with the design outlined in this protocol and to abide by all provisions of this protocol (including other manuals and documents referenced from this protocol).

I agree to conduct the study in accordance with generally accepted standards of Good Clinical Practice (ICH-GCP), the ethical principles of the Declaration of Helsinki.

I also agree to report all information or data in accordance with the protocol and, in particular, I agree to report any serious adverse events as defined in the protocol. I agree to handle all clinical supplies provided by the Sponsor (or their designate) and collect and handle all clinical specimens in accordance with the protocol and other manuals and documents referenced from this protocol.

Since information in this protocol and associated documents is confidential, I will ensure the necessary precautions are taken to protect such information from loss, inadvertent disclosure, or access by unauthorised third parties.

Matthias Schmuth (Department of Dermatology, Medical University Innsbruck)
University Clinic for Dermatology, Venereology and Allergology Anichstr. 35 6020 Innsbruck Austria Tel: […] Fax: […] E-mail: […] _____ Date, signature
NN (Department of Dermatology, Medical University Innsbruck)
Innsbruck University Clinic for Dermatology and Venereology Anichstrasse 35 A-6020 Innsbruck Austria Phone: […] Fax: […] E-mail: _____ _____ Date, signature
NN **(Department of Dermatology, Medical University Innsbruck)** Innsbruck University Clinic for Dermatology and Venereology Anichstrasse 35 A-6020 Innsbruck Austria Phone: […] Fax: […] E-mail: _____ _____ Date, signature

Questionnaire on the health risk culture model

Scale: How important / relevant is the following in relation to your own health?

Visual analogue scale / slider from 0 - 100

Dimension	Factors	Items
Person observable	Routines	My fixed habits / routines
		That I (don't) eat meals regularly
		That I (don't) exercise regularly
		That I (don't) sleep at fixed times
		That I (don't) have habits when consuming stimulants (sweets, coffee, etc.)
		That I (don't) have habits when it comes to hygiene (showering, washing, brushing teeth, etc.)
		That I (don't) go to the doctor regularly
	Socio-Demographics	My gender
		My age My profession / vocational training
		My educational qualifications
		My relationship status
		My current stage of life (e.g. the last two months)
		My financial situation
		My social environment
	Risk behaviour	That I (do not) smoke
		That I (do not) consume stimulants (sweets, coffee, etc.)
		That I (do not) exercise or (do not) do sport
		That I (do not) consume alcohol
		That I (do not) consume drugs
		That I (do not) consume prescription drugs without a doctor's prescription
		That I (do not) have unprotected sex with changing partners That I (do not) sit for long periods
		My daily diet
		My work situation
		My driving behaviour in traffic

	Judgement & decision	My planned decisions
		My spontaneous decisions
		That I relate health risks & consequences to myself
		That I relate health risks & consequences to others
		That I (do not) comply with treatments / medications
		That I am (not) able to make good decisions.
	Physical state	That I have (no) complaints due to physical illnesses
		That I have (no) mental illnesses / complaints
		That I am (not) able to work
		That I have (no) impairments in my lifestyle due to pain / complaints
		My sleep My
		energy (physical, mental)
		My stress
		My worries and fears
Person nonobservable	Risk perception, awareness & attitude	My own quality of life
		That I am (not) aware of my health
		That I am (not) aware of health risks
		That I (not) trust experts / authorities
		That I (not) recognise health threats at an early stage
	Values & beliefs	That I am (not) confident / optimistic
		That I am (not) convinced that I live in a just world
		That I am (not) convinced that external factors are responsible for my success / failure
		That I am (not) convinced that I am responsible for my own success / failure
		That I am (not) convinced that I deserve my health
		That I am (not) convinced that I can influence my own health / illness
		That I am (not) convinced that I am protected (e.g. by a higher power / God)
	Experience, knowledge & competence	My own knowledge and my own experiences
		That I feel (no) belonging
		That I feel (no) autonomy
		That I feel (no) controllability
		That I feel (no) hope
		That I feel (no) helplessness
		That I have had (no) particularly formative or drastic experiences
		That I am (not) robust in the face of crises That
		I have (no) health-promoting talents (e.g. athleticism, talent in cooking, etc.)
		That I have (not) had the experience of being able to influence my own health

	Personality	My own personality
		My own temperament
		My willingness to take risks
		My degree of impulsiveness
		That I (do not) strive for borderline experiences
		That I am (not) socially acceptable
		That I am (not) conscientious
		That I am (not) reasonable
		That I am (not) carefree
		That I am (not) emotionally stable
		That I am (not) open and approachable
		That I am (not) open to new things
		That I (do not) avert risks early on
	Self-efficacy & feelings	That I (do not) feel competent & capable
		That I (do not) have the feeling that I can control & regulate myself
		That I (do not) have the feeling that I can solve problems independently & reliably
		That I am (not) sure that I can solve possible difficulties.
Context observable	Composition	The gender of the people in my social groups
		The number of people in my social groups
		The age of the people in my social groups
		The professional activities of the people in my social groups
	Hierarchy & leadership	My care situation through systems (e.g. health insurance, employer, medical care at work, etc.)
		That I have (no) private health motivators (e.g. friends who encourage me to eat healthily) That
		I have (no) professional health motivators (e.g. fitness coaches, trainers, etc.)

		That I (do not) receive help from others (e.g. parents, family, partner, etc.) with my health care
		That I (do not) take care of my health independently
	Narratives & legends	My family illness stories
		My family health/healing stories
		The health-related experiences of my family members/acquaintances
		The health-related measures taken by my employer
	Roles & responsibility	That I (do not) see myself in an active role with regard to my health
		That I (do not) behave in a gender-typical way
		That I (do not) behave according to my self-image
		That I (do not) behave in a way that is typical for my current stage of life (e.g. the last two months)
		That I (do not) treat my health selflessly
	Structures	That I receive (no) information from the media
		That I receive (no) information from my environment
		That I receive (no) information from health professionals (doctors, therapists, counsellors, etc.)
		That I have (no) health-promoting programmes & initiatives in my environment
		That I have (no) extensive and appealing sports offers in my environment
		That I have (no) extensive and appealing food supply
		My insurance cover
		The processes at my workplace
Context nonobservable	Norms & social identity	That I (do not) behave in accordance with the group
		That I (do not) stand out from others through my behaviour
		That I (do not) feel part of my social group
		That the values and rules of my social group are (not) also my values and rules
		That the social role I want to play is (not) reflected in my behaviour

	Values & beliefs	That I (do not) differ from the health/risk behaviour of my social groups That my parents/acquaintances (do not) exemplify health-related values & beliefs That I (do not) orientate myself towards scientific & health-related knowledge That I (do not) take care of my health myself That other people (do not) take care of my health
	Trust	That I have (no) trust in doctors & paramedics That I have (no) trust in health insurance companies & social systems That I have (no) trust in help from fellow human beings That I have (no) trust in producers of my food That I have (no) trust in existing health knowledge
	Goals & expectations	That I (do not) set myself health-related goals & expectations That I (do not) achieve my health-related goals & expectations That I (do not) have the goal of a long and healthy life That I (do not) have the goal of being recognised in social relationships That I (do not) have the goal of being important to others That I (do not) have the goal of being reliable in social relationships That I (do not) have the goal of showing solidarity in social relationships That I (do not) have the goal of being autonomous in social relationships That I (do not) have the goal of finding protection and boundaries in social relationships
	Shared experience & group history	That I (do not) share health-related experiences with others That I (do not) have health-related experiences with others That I (do not) orientate myself towards the health-related behaviour of people who are important to me
Risk situation observable	Domain	That I (do not) take risks where I have fun That I (do not) take risks where I receive recognition That I (do not) take risks where I reduce stress That I (do not) take risks where I feel a zest for life That I (do not) take risks where I realise myself Natural disasters and climate change My everyday risks My one-off special risks

	Potential outcomes & severity	That I am (not) aware that I can be hurt by my behaviour That I am (not) aware that I can become ill through my behaviour That I am (not) aware that I can die through my behaviour That I am (not) aware that I can be ostracised through my behaviour That I am (not) aware that I can receive recognition through my behaviour That I am (not) aware that I can become healthier through my behaviour That I am (not) aware that I can become happier through my behaviour That I am (not) aware that I can receive recognition through my behaviour That I am (not) aware that my behaviour could make me healthier That I am (not) aware that my behaviour could make me happier That I am (not) aware that my behaviour could change my weight
	Psychological distance	That I (do not) take timely health precautions That I (do not) get vaccinated early That I (do not) go to health screenings early That I (do not) take health measures that are effective now That I (do not) take health measures that will be effective in the future
	Framing	My own behaviour The behaviour of my social environment My own body (genetics, physiology, etc.) Many small aspects / measures to improve my health Few large aspects / measures to improve my health That I (do not) influence my health myself That fate / chance has (no) influence on my life That I (do not) try to become healthier That I (do not) try to become ill
Risk situation nonobservable	Likelihood	That I (do not) know the probability of becoming ill () That I (do not) know the probability of my own death () That I (do not) know the probability of staying healthy () That I (do not) know the probability of my own injury That I (do not) know the probability of side effects (medication / treatments) That I (do not) know the probability of successful treatment That I generally (do not) orientate myself towards probabilities

Uncertainty	That I (do not) have my own rules or rules of thumb for my behaviour That I (do not) ignore uncertainties
	That I assume a secure / uncertain future That I (do not) adapt my behaviour in confusing situations
Complexities	That I (do not) engage with health information, therapies, etc.
	That I (do not) question health programmes / interventions
	That I (do not) assume that my health-related ideas are generally valid
	That I (do not) implement my own health-related assumptions in my behaviour
Emotionality	My situational emotions (e.g. anger, frustration, joy, etc.)
	My general emotional tendencies (e.g. reactions typical for me)
	My situational impatience / hecticness
Ambiguities	That I feel a (/no) conflict between desired health behaviour (e.g. sport, eating, etc.) and opposing motives (e.g. fun, comfort, etc.)
	That I (do not) behave in a way that fits in with my desired health behaviour
	That I have (no) difficulty in adapting my behaviour to my health expectations (e.g. stop smoking, lose weight)