**Project Title: Reverse the clock: A research project of collagen activator effects on biological age and skin quality**

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| Research legislation: | Ordinance on human research with the exception of Clinical trials (HRO) [1]. |
| Type of Research Project: | Research project involving human subjects |
| Risk Categorisation: | Risk category A acc. to ordinance HRO Art. 7 |
| Project leader: | Dr. med. C. Bettina Rümmelein, Medical leadership at Hautwerk dermatology clinic Zürich, Maneggstr. 17, 8041 Zürich, +41 43 343 93 01, ruemmelein@hautwerk.ch |

**Protocol Signature Form**

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| Study Title | Reverse the clock: A research project of collagen activator effects on biological age and skin quality |

The project leader has approved the protocol version 4 ***(dated 29.08.2023)***, and confirms hereby to conduct the project according to the protocol, the Swiss legal requirements [1,2] , current version of the World Medical Association Declaration of Helsinki [3] and the principles and procedures for integrity in scientific research involving human beings.

**Project leader:**

Site: Hautwerk *[Maneggstr. 17, 8041 Zürich]*

Name: Frau Dr. med. C. Bettina Rümmelein

Date: 29.08.2023 Signature: 

**Sponsor:**

Name: Sophie Chabloz, representing Avea Life AG

Date: 29.08.2023 Signature: 

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**GLOSSARY OF ABBREVIATIONS**

| | |
|---|---|
| *BASEC* | *Business Administration System for Ethical Committees* |
| *CRF* | *Case report form* |
| *FOPH* | *Federal Office of Public Health* |
| *HRA* | *Human Research Act* |
| *HRO* | *Ordinance on Human* |

## 1. BACKGROUND and project rationale

Skin aging is a complex process that involves both intrinsic and extrinsic factors. Intrinsic factors, such as genetic and hormonal changes, play a role in skin aging, while extrinsic factors, such as exposure to UV radiation and environmental toxins, accelerate the aging process. The signs of skin aging include wrinkles, age spots, reduced elasticity, reduced hydration, and increased transepidermal water loss [4,5].
As we age, the body's natural ability to replenish collagen decreases by approximately 1.0%-1.5% per year. This decline in collagen production is a significant factor contributing to the development of fine lines and deeper wrinkles. Additionally, within the deeper layers of the skin (dermis), essential components of the extracellular matrix such as fibrillar collagens, elastin fibers, and hyaluronic acid undergo noticeable structural and functional changes [5,6].
The aging process brings about various changes in the different layers of the skin. In the outermost layer, the epidermis, the thin basal lamina that separates it from the dermis becomes thinner, resulting in reduced cell turnover and a thinner epidermis. This leads to decreased barrier function, slower recovery, increased water loss, reduced hydration, and increased stiffness [7].
The connection between the epidermis and the dermis, known as the dermal-epidermal junction, weakens with age, contributing to wrinkle formation and further thinning of the epidermis. In the dermis, the second layer of the skin, significant changes occur. Collagen, the predominant protein, undergoes increased fragmentation, leading to fewer fibroblasts and reduced production of type I collagen. Additionally, collagen degradation by matrix metalloproteinases (MMPs) increases, resulting in dermal thinning and the formation of wrinkles [7].
Aging also affects elastin, another crucial protein in the skin. Exposure to UV radiation enhances elastin degradation, while reduced synthesis by fibroblasts leads to decreased skin elasticity. Hyaluronic acid, which plays a vital role in skin hydration, experiences a reduction in synthesis, leading to decreased skin hydration [7].
In conclusion, skin aging is a complex process involving changes in the epidermis, dermal-epidermal junction, and dermis. The signs of skin aging, including wrinkles, age spots, reduced elasticity, reduced hydration, and increased transepidermal water loss, are the result of these changes. Understanding the molecular and cellular mechanisms underlying skin aging can help in the development of effective anti-ageing strategies [5,7].

Epigenetic methylation clocks are emerging as a promising biomarker of biological age. DNA methylation is a chemical modification of DNA that regulates gene expression and plays a critical role in development and aging. Methylation patterns change over time, and researchers have developed epigenetic clocks based on DNA methylation patterns to estimate biological age [8–12].
Accelerated epigenetic clocks, which are associated with older biological age, have been linked to several health outcomes. Studies have shown that accelerated epigenetic clocks are associated with mortality, cardiovascular disease, cancer, diabetes, and higher BMI [13–17].
Intervention studies have used epigenetic clocks to show a reduction in biological age. For example, diet and lifestyle interventions have been shown to reduce biological age, as measured by epigenetic clocks [18–20]. Additionally, supplementation with alpha-ketoglutarate has been shown to decrease biological age [21]. These findings suggest that epigenetic clocks may be useful in evaluating the efficacy of interventions aimed at improving health and longevity.
Our trial provides an opportunity to further establish the correlation between epigenetic clocks and relevant health measures. By measuring DNA methylation patterns and biological age in our participants, we can evaluate the relationship between epigenetic clocks and health outcomes, in our case multiple measures of skin aging. These findings may provide insights into the mechanisms underlying aging and identify new targets for interventions aimed at improving health and longevity.

Skin measurements are essential tools for evaluating the efficacy of interventions aimed at improving skin health and appearance. The Corneometer is a commonly used device for measuring skin hydration. Several studies have shown that oral collagen peptide supplements can improve skin hydration, as measured by the Corneometer, over a period of two to three months [22–28]. However, one study found no improvement in skin hydration with collagen peptide supplementation [29].
Skin elasticity is another important measure of skin health. The Cutometer is a device commonly used to measure skin elasticity. Studies have shown that oral collagen peptide supplements can improve skin elasticity, as measured by the Cutometer, over a period of two to three months [22–31]. However, one study found no improvement in skin elasticity with marine collagen supplementation [32].
Skin thickness is also an important measure of skin health. Ultrascan is a non-invasive method for measuring the thickness of the epidermis and dermis, as well as collagen density. Studies have shown that oral collagen peptide supplements can improve skin thickness, as measured by sonography, over a period of two to three months [24,30].
Overall, these skin measurements provide valuable information on the efficacy of interventions aimed at improving skin health and appearance. Oral collagen peptide supplements have been shown to improve skin hydration, elasticity, and thickness in several studies. However, some studies have found no improvement in skin hydration or elasticity with collagen peptide supplementation. These inconsistencies may be due to differences in the type, dose, and duration of collagen peptide supplementation, as well as individual variations in response to supplementation.

In addition to the information provided, Visia skin analysis is another widely used tool for evaluating skin health and appearance. Studies have shown that oral marine collagen [32] and hydrolyzed collagen supplements [23] can lead to a reduction in wrinkles over a period of two to three months, as measured by Visia skin analysis. The effectiveness of collagen peptide supplements in improving skin health and appearance has also been evaluated using the Miravex Antera 3D system, which provides a comprehensive analysis of skin texture, pigmentation, and vascularization.
Several studies have been conducted to validate the Visia system, including those by Hensler et al. [33–35] and Wang et al. [36]. These studies have demonstrated the reliability and accuracy of the Visia system in measuring skin parameters such as wrinkles, pigmentation, and pore size.
Overall, these skin measurement tools provide valuable information on the effectiveness of collagen peptide supplements in improving skin hydration, elasticity, thickness, and reducing wrinkles and other signs of aging. However, it is important to note that individual variations in response to supplementation may exist, and further research is needed to determine the optimal type, dose, and duration of collagen peptide supplementation for improving skin health and appearance.

The justification for categorizing the clinical trial involving a *collagen activator* and non-invasive measurements as risk category A is based on the fact that the intervention is considered low risk and has a well-established safety profile. The *collagen activator* supplement is freely available on the market. Participants are taking the *collagen activator* voluntarily and the potential risks associated with taking the supplement are minimal, with no known serious adverse effects reported.
In addition, the measurements involved in the study are all non-invasive and have been widely used in clinical research. The use of the corneometer, cutometer, ultrascan, and Visia scan are considered standard techniques for measuring skin hydration, elasticity, thickness, density, pigmentation, wrinkles, texture, UV-spots, pores, redness, and porphyrins. These measurements are safe and pose no known risks to participants when used by trained personnel.
Furthermore, the spit test used to measure biological age with an epigenetic methylation clock is also non-invasive and has been used in previous clinical trials. The use of this test

poses no known risks to participants and is a well-established technique for measuring biological age.
Therefore, based on the low-risk nature of the intervention and the non-invasive measurements involved, this clinical trial can be justified as risk category A.

## 2. project OBJECTIVES and Design

### 2.1 Hypothesis and primary objective

Primary objective: Intake of the collagen activator from Avea for 6 months will result in a significant decrease in biological age compared to the baseline.
Secondary objective: Intake of the collagen activator from Avea will lead to a significant improvement in skin quality and texture, including increased hydration, elasticity, and reduction in wrinkles, pigmentation, texture, UV-spots, pores, redness, and porphyrins after 3 months compared to baseline.

### 2.2 Primary and secondary endpoints

The primary endpoint of our trial is to assess changes in DNA methylation patterns as a biomarker of biological age after the intake of the collagen activator from Avea for 6 months. Epigenetic clocks, based on DNA methylation patterns, have been shown to be associated with various health outcomes such as mortality, cardiovascular disease, cancer, diabetes, and higher BMI in several studies including Lau et al., McCrory et al., Föhr et al., Oblack et al., and Lo et al.[13,15–17,37].
Therefore, the rationale for using DNA methylation patterns as a biomarker of biological age is to investigate whether the intervention with the collagen activator can slow down the accelerated epigenetic clocks and potentially reduce the risk of age-related health conditions. Previous intervention studies using epigenetic clocks have shown promising results in reducing biological age through dietary and lifestyle interventions, alpha-ketoglutarate, and other lifestyle interventions, as demonstrated by Fitzgerald et al., Demidenko et al., Duan et al., Fiorito et al., and Pavanello et al.
In addition, our trial aims to establish the correlation between epigenetic clocks and relevant skin health measures. To control for potential baseline factors that might influence the outcome, we will consider factors such as gender, age, chronic diseases and smoking. By investigating changes in DNA methylation patterns, our trial will provide valuable insights into the efficacy of the collagen activator in improving cellular and tissue health and potentially reducing the risk of age-related health conditions.

Secondary endpoints:

- Hydration (measured with the corneometer): Baseline factors we consider that can influence hydration levels are age, gender, and skin type. Adequate hydration is important for maintaining skin health and preventing dryness and roughness.
- Elasticity (measured with the cutometer): Baseline factors we consider that can influence skin elasticity are age, sun exposure, and smoking. Good skin elasticity is a sign of healthy, youthful skin.
- Skin thickness and density (measured with the ultrascan): Baseline factors we consider that can lead to thinning of the skin are age and sun exposure. Measuring skin thickness and density can help determine the overall health of the skin.
- Pigmentation, wrinkles, texture, UV-spots, pores, redness, and porphyrins (measured with the Visia scan): Baseline factors we consider that can influence these factors are age, sun exposure, smoking, and genetics. These measurements can help determine the overall appearance and health of the skin.

These measurements contribute to determining skin quality and texture by providing objective and quantitative data on various aspects of the skin. By monitoring changes in

these measurements over time, researchers can assess the effectiveness of the collagen activator aimed at improving skin quality and texture.

### 2.3 Project design

The study is a human research study in which customers that are taking the collagen activator from Avea are observed for six months. The trial is in the form of a post marketing surveillance observational trial since the product has already been approved and is sold on the market. All participants are voluntarily taking the collagen activator. There is no blinding, randomization or placebo control that is taking place in the trial. The results are compared individually for each participant after the follow ups from their baseline measurements.

- The trial exposure is the Collagen activator from Avea
    - Potential participants are contacted after purchasing the collagen activator for 6 months via Avea's online shop. After successful recruitment they undergo the baseline assessment. They are instructed to only start taking the collagen activator once the baseline assessment is completed.
    - Daily oral intake of 1 package of 11.5g diluted in water over six months, taken in the morning
- Saliva samples are taken twice (time points 0 and +6 months) to determine biological age via epigenetic methylation clock (Trume labs are the test providers and analysers)
- Skin analysis takes place at time points 0, +1 and +3 months to determine skin quality. This includes measurements of hydration, elasticity, skin thickness and density, as well as pigmentation, wrinkles, texture, UV-spots, pores, redness and porphyrins.
- Skin tests used for this are the corneometer, cutometer, ultrascan and Visia scan.
- Every month the participants receive a questionnaire regarding recent subjective changes, adverse reactions and adherence

The individual duration expected for each patient from the recruitment process onwards is about 6-7 months

- The goal is to recruit 60 healthy participants that are older than 35 years and are voluntarily taking the collagen activator for 6 months
- 2-3 weeks from being recruited to receiving a date for the baseline measurements
- Day 1 being the day of the baseline measurements
- Follow ups for the skin measurements take place +1 and +3 months from day 1
- Follow up for the saliva test takes place +6 months from day 1

The justification for this study design lies in its ability to provide a comprehensive assessment of skin quality and biological age. By measuring a range of skin quality parameters, the study will be able to capture changes in skin texture and appearance over time. Similarly, by measuring biological age using an epigenetic methylation clock analysis, the study will be able to capture changes in biological age over time.
Overall, the use of a combination of skin measurements and biological age measurements provides a robust and comprehensive approach to measuring changes in skin quality and biological age over time with the use of the *collagen activator* by Avea.

To account for the influence of women's menstrual cycle on the skin measurements, the study doctor will ask female participants at each visit about the first day of their most recent menstrual period. This is a standard procedure to determine if a women is in the follicular phase (first 2 weeks from the first day of period) or in the luteal phase (more than 2 weeks from the first day of period). This will be accounted for during the data analysis to reduce confounding. Further, as both females and males are included in this study, sex will be

included as a covariate in the analysis to address potential differences between male and females.

Limitations:
We do not have the means to collect direct data about UV-exposure and try to account for it within the baseline questionnaire. We ask about the usage of sunscreen of participants, specifically how regularly they apply it and which sun protection factor they use, as well as how much time they usually spend in the sun. This will then be considered in the data analysis. Further, it is not possible to do all the skin analyses at a consistent time of day.

## 3. PROJECT POPULATION and Study procedures

### 3.1 Project population, inclusion and exclusion criteria

The project population consists of healthy adults over the age of 35 years, regardless of gender or ethnicity, who are willing and able to give their informed consent to participate in the study. The total number of participants will be 60. The choice of participants over 35 years is because in younger individuals collagen is not yet depleted or only to a small extent [5]. To ensure gender balance in the recruitment, our inclusion criteria are gender-neutral, gender-neutral language is used in all study materials and advertisements and we will engage in diverse recruitment to target a broad population.

Participants fulfilling the following inclusion criteria are eligible to participate in the study:

- Age 35 or older, regardless of gender, ethnicity or socioeconomic background
- Voluntarily purchasing the collagen activator (from Avea) for 6 months with the intend to take it on a daily basis
- Willing to fill out the questionnaires on a monthly basis.
- Willing to participate in all measurements part of the study (Skin tests & saliva samples)
- Able to appear at the baseline, 1 and 3 month follow up visits at the Dermatology clinic “Hautwerk” in Zürich.

If any of the following exclusion criteria are present, the participant will be excluded from the study:

- Underlying health conditions leading to regular intake of medication, including:
    - Diabetes
    - Hypertension (high blood pressure)
    - Cardiovascular disease
    - Asthma
    - Chronic obstructive pulmonary disease (COPD)
    - Arthritis
    - Depression
    - Anxiety
    - Epilepsy
    - Cancer
    - Thyroid disorders
    - Autoimmune diseases such as lupus or multiple sclerosis
    - Chronic kidney disease
    - Liver disease
    - HIV/AIDS
    - Chronic pain conditions such as fibromyalgia or chronic migraine.
    - …

- Chronic skin condition, independent of treatment, including:
    - Acne
    - Eczema
    - Psoriasis
    - Rosacea
    - Skin cancer
    - …
- Intake of any collagen supplements 3 months prior to the screening date
- Smoking at time of recruitment or smoking up to 5 years prior to recruitment
- Pregnancy or lactating at the time of recruitment or planning to get pregnant in the following 6 months
- Insufficient knowledge of the German or English language
- Unable to give consent
- Unable to follow the procedures that are part of the study

**3.2 Recruitment, screening and informed consent procedure**

Recruitment will be conducted through advertising the study to Avea customers after they have bought the collagen activator in the Avea online shop for the first time. The advertisement will be uploaded in BASEC.

Potential participants are Avea customers, therefore the Avea team has access to their email addresses. The study advertisement is sent to the email address under which the order was placed. The first round of pre-selection will be done by the potential participants themselves by going through the study advertisement and the description of the trial. If a person decides they are interested in participating, they can fill out the inclusion and exclusion questionnaire which is attached to the advertisement (via a link to a google form). In this form they enter their email address. For questions they can reach out via email to the person in charge of recruitment at Avea. The results of the google form are only visible to the Avea team member in charge of recruitment. Once that person has decided if a person is eligible to participate according to the criteria defined in section 3.1 (if uncertain the decision is made together with Dr. Rümmelein), the answers of the google form are deleted immediately and the email address is transferred to RedCap. From then on they are considered as participants and all information is securely stored and encrypted in RedCap. Via RedCap they then receive the detailed study information. This entails the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits and any discomfort it may entail. Each subject is informed that the participation in the study is voluntary and that he/she may withdraw from the study at any time and without stating any reason. The withdrawal of consent will not affect his/her subsequent medical assistance and treatment. The subjects are informed that he/she can ask any question, and consult with family members, friends, their treating physicians or other experts before deciding about their participation in the study. Enough time is given to the subjects to make a decision and to give informed consent at all times. The subjects are informed that authorized individuals may examine his/her medical records and how and for which purpose the extracted data will be used.

Together with the detailed study information they receive a booking link from the Hautwerk clinic (only for the study) where they can schedule their first appointment (within the following 3 weeks) if they remain willing to participate. They can schedule a phone call with Dr Rümmelein to clarify any remaining questions (optional they can also reach out to her via email if there are only a few or no questions).

At the first appointment, each subject will once again fill out the inclusion and exclusion questionnaire to confirm their eligibility for the study. Dr. Rümmelein will explain again to each subject what it means to be part of the study (see paragraph above), and will fill out the informed consent form with each participant.

The participants should read, understand, and voluntarily agree before signing and dating the informed consent form, and are given a copy of the signed document. The consent form is signed and dated by the participant and the Project leader Frau Dr. Rümmelein, or her assigned substitute. The signed consent form is retained as part of the investigation records.

No direct payment is given to the study participants. Each person does receive the skin scans and results of the saliva biological age tests after completion of the study free of charge (information only provided after completion of the study and if the participant has not informed us of not wanting to receive this information).

### 3.3 Study procedures

The recruitment period will presumably start in August 2023 and the latest follow-up visit for a participant is expected to be in December/January 2023. For an individual participant, the recruitment period will be 1 month or shorter and includes an online screening procedure and phone call with Frau Dr. Rümmelein or her team. Participants will be informed about all study details as described in 3.2 via email and time will be given to them to make a decision about the participation and to give informed consent at all times. In case of any questions, they can reach out to the person in charge of recruitment via phone. After successful completion of the screening, a first visit at the Dermatology clinic Hautwerk in Zurich will be scheduled as soon as possible (not later than a month). The participants will receive an online medical questionnaire (via REDcap), which they can fill out at home before the first visit if they are able to, or if not they will fill it out at the first visit under supervision of a medical professional (doctor). At the first visit Dr. Rümmelein will go through all the relevant information again with the participants which allows them to give their informed consent and will fill out the informed consent form with them. The participants receive a copy of the signed form.

At the first visit at the Dermatology clinic Hautwerk (time point 0), the medical professional will then check if the participant has already filled out the medical questionnaire. If yes, they will go through the most important points to check the participant understood everything correctly, if no, they will supervise the participant in filling out the questionnaire. After the questionnaire is completed, the medical professional will instruct the participant to take a saliva sample via spitting on a spoon and transferring the saliva to a piece of paper (provided by TruMe laboratories). The paper has to dry for 45 minutes in the open air (taken care of by the medical professional). Then, the medical professional performs the non-invasive skin scan (Visia analysis system), and the tests with the corneometer, cutometer and ultrascan. Afterwards, there will be time for the participant to rest until it can be sure that there are no adverse reactions to any of the procedures performed. The whole visit will be recorded by the medical professional. The total visit should take between 30 to 60 minutes. The saliva samples are stored without risk at ambient temperature. The study personnel will collectively ship the samples to Trume labs once all the baseline visits are completed.

Specifics to the skin measurement procedures:

1. Cutometer: The cutometer is a non-invasive device that measures the elasticity of the skin. It uses suction to apply a negative pressure on the skin, which causes the skin to deform. The device then measures the time it takes for the skin to return to its original position after the suction is released. This measurement is known as the skin's viscoelastic properties, and it is used to assess the skin's firmness and elasticity.
2. Corneometer: Corneometer is a non-invasive device that measures the moisture content of the skin. It works by applying a small electrical current to the surface of the skin, which measures the skin's electrical impedance. The device then calculates the moisture content of the skin based on the electrical impedance. This measurement is used to assess the hydration level of the skin.

3. Ultrascan: Ultrascan is a non-invasive device that uses high-frequency ultrasound waves (22 Mhz) to measure the thickness and density of the skin. The device works by emitting ultrasound waves into the skin, which bounce back and are recorded by the device. The time it takes for the ultrasound waves to bounce back is used to calculate the thickness of the skin. This measurement is used to assess the overall health of the skin and collagen content can be read out by it.
4. Visia scan: Visia scan is a non-invasive imaging system that captures high-resolution images of the skin's surface. The device uses different types of light, such as UV, polarized, and non-polarized light, to capture images of the skin at different depths. The images are then analyzed to assess various skin conditions, including pigmentation, wrinkles, texture, UV-spots, pores, redness, and porphyrins. This measurement is used to assess the overall health and quality of the skin.

At the time points +1, +2, +3, +4 +5 and +6 months of the study duration, a short online questionnaire will be sent to all participants via email, with a secure link to the REDcap online form. It will contain questions about the product intake (how many days of the months), the health status (any changes, physical or mental illness, adverse reactions), important lifestyle changes, and if the participants are still willing to continue with the study. There will also be the possibility for the participants to reach out to a medical professional or to schedule a visit.

At the timepoints +1 and +3, follow-up visits at the Dermatology clinic Hautwerk will be scheduled (time frame +/- 1 week of the +1 and +3 months time point respectively). The visit will contain the same procedures as the visit at time point 0, minus the saliva sample. The medical professional will again first check the completion of the questionnaire and assist if necessary, then take the skin scan (Visia analysis system), and the tests with the corneometer, cutometer and ultrascan. The participants have then time to rest to make sure there are no adverse reactions to any of the procedures. At time point +3, the participant will be provided with the saliva collection kit for timepoint +6 months. Each visit will be recorded by the medical professional. The total visit should take between 30 to 60 minutes.

At the time point +6 months, a second saliva sample will be collected (time frame +/- 1 week of the +6 months time point). Participants will have received the collection kit at time point +3 by the study personnel. They will be reminded 1 week prior to the due date of the test and 1 day before, and will be informed about the exact procedures on how to perform the test and have the possibility to ask any questions to the study personnel via phone. They have to spit on a spoon and put the saliva on the provided paper and let it air dry for 45 minutes, before sending it to the investigators site (Avea Life, Bahnhofplatz Zug). There the samples will be collectively sent for analysis to Trume labs once all samples from time point +6 have been collected. Storage of the samples is without risk at ambient temperature.

To minimize bias in the study population, during the recruitment process a focus will lie on individual participant attributes that could confound the data like gender, ethnicity and age and there will be efforts made to generate a broad spectrum of study participants to avoid only collecting information from one subgroup of the population. Also, all tests (saliva, skin analyses), and questionnaires are performed on all study participants and for the same duration of time. In the questionnaire we will ask for potential confounders so that we can account for them in the data analysis (e.g. being sick during study period, major changes in behavior etc.). The skin analyses will all be performed by the same person (doctor) to reduce measurement bias.

### 3.4 Withdrawal and discontinuation

The Sponsor or Projectleader may terminate the trial prematurely for individual participants or the participants may withdraw from the study according to certain circumstances:

- Participants can withdraw from the study if they have any concerns regarding their health or safety being compromised due to the procedures during the study or if they voluntarily stop to take the collagen activator, which no longer qualifies them to be part of the study.
    - This includes any adverse reactions to the procedures during the study
    - Other restrictions or reductions regarding their mental or physical health that is linked to the procedure during the trial
    - if they withdraw their informed consent
    - if they voluntarily decide to stop taking the “intervention”
- Participants can be excluded from the trial in case of non-compliance
    - Incorrect / irregular intake of the “intervention”
    - Failing to regularly answer the questionnaire
    - Missing out or not showing up to the appointments
    - Not adhering to the required testing procedures

In case a participant has to withdraw or be excluded from the study, their data will not be included in the analysis (sample size is adjusted for a drop-out rate of 15%). There will be no final examination of the participant and the previously gathered material of the participant will be destroyed.

## 4. STATISTICS AND METHODOLOGY

### 4.1. Statistical analysis plan

In this project the null-hypothesis for the primary endpoint is the following: There is no significant reduction in biological age compared to chronological age after 6 months of collagen activator supplementation. The alternative hypothesis is the following: There is a significant reduction in biological age compared to chronological age after 6 months of collagen activator supplementation. To determine the sample size, the following power analysis was conducted using the “pwr” package in R for a one-sided hypothesis: The power of the test is set at 0.8, the significance level is set at 0.05 and the effect size at 0.5. This results in a sample size of n=50. This was then adjusted for an estimated drop-out rate of 15-20%, which results in a sample size of n=59-63. Therefore, we set the sample size at n=60.

To accommodate the inclusion of sex as a covariate in the statistical analysis, the primary endpoint analysis will employ a linear regression model. The model will include the following variables:

- Biological age reduction as the dependent variable.
- Chronological age as a covariate to account for its potential influence.
- Sex as a categorical covariate to control for the effects of gender.

The analysis will utilize the "lm" function in R's "stats" package. The significance level will be set at 0.05 (one-sided). Effect size will be calculated using Cohen's d. To correct for multiple testing, the p values will be adjusted with the Benjamin-Hochberg procedure.

Secondary endpoints within this study will be subjected to similar analysis procedures, employing linear regression models that incorporate sex as a covariate, while retaining the paired t-test structure for comparing pre- and post-intervention results.

In conclusion, the inclusion of sex as a covariate in the analysis allows us to control for its effects without altering the originally determined sample size. This approach enhances the accuracy of our analysis by considering potential sex-based variations in the primary endpoint while adhering to rigorous statistical practices.

### 4.2. Handling of missing data

In case of missing data, this will be handled with an “only complete case analysis”. This involves only analyzing data from participants who have completed all study visits and provided all required data. In the sample size calculation we compensated for expected drop-outs (rate of 15%).

## 5. Regulatory Aspects AND SAFETY

### 5.1 Local regulations / Declaration of Helsinki

This research project will be conducted in accordance with the protocol, the Declaration of Helsinki [3], the principles of Good Clinical Practice, the Human Research Act (HRA) and the Human Research Ordinance (HRO) [1] as well as other locally relevant regulations. The project leader acknowledges his responsibilities as the project leader

### 5.2 Notification of safety and protective measures (HRA Art. 15, HRO Art. 20)

If, during the research project, circumstances arise which could jeopardize the safety or health of the participants or lead to a disproportionate relationship between the risks and burdens and the benefits, all the measures required to ensure protection are to be taken without delay.
The Principal Investigator and the Sponsor are promptly notified (within 24 hours) if immediate safety and protective measures have to be taken during the conduct of the research project. The Ethics Committee will be notified via BASEC of these measures and of the circumstances necessitating them within 7 days.

### 5.3 Serious events (HRO Art. 21)

If a serious event occurs, the research project will be interrupted and the Ethics Committee notified on the circumstances via BASEC within 7 days according to HRO Art. 21.
„A Serious Adverse Event (SAE) is classified as any untoward medical occurrence that:
- results in death
- is life-threatening
- requires in-patient hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability/incapacity, or
- is a congenital anomaly/birth defect.

### 5.4 Procedure for investigations involving radiation sources

No radiation sources are being used in this project.

### 5.5 Amendments

Substantial changes to the project set-up, the protocol and relevant project documents will be submitted to the Ethics Committee for approval according to HRO Art. 18 before implementation. Exceptions are measures that have to be taken immediately in order to protect the participants.

### 5.6 End of project

Upon project completion or discontinuation, the Ethics Committee is notified within 90 days.

### 5.7 Insurance

In the event of project-related damage or injuries, the Sponsor will be liable, except for damages that are only slight and temporary; and for which the extent of the damage is no greater than would be expected in the current state of scientific knowledge (Art. 12 HRO).

## 6. FURTHER Aspects

### 6.1 Overall ethical considerations

Overall ethical considerations of the project:
The primary ethical consideration of this study is ensuring the generalizability and overall social and scientific value of the project. To achieve this, the study will be conducted in healthy adults of both genders and different ethnicities. By ensuring that the study design and procedures are appropriately robust, and that the participant cohort is diverse and representative, the results of the study can be used to inform future research and decision-making in a meaningful and impactful way.
All procedures have been thoroughly justified, and all participants will be provided with clear information regarding their rights, risks, and obligations.
Incidental findings relevant to a person's health will be communicated to all participants, unless they inform us that they do not wish to be informed. They are made aware of this in the study information. Individual study results will be communicated to all participants once the whole study is completed, unless they inform us that they do not wish to be informed. They are made aware of this in the study information.
All participants are informed about the special risks associated with genetic data. In this study saliva samples are collected to determine biological age. For this epigenetic methylation is analyzed as well as 9 short DNA sequences. The saliva samples as well as the DNA sequence data is deleted after the determination of biological age and is not used to generate any health related information of the participants.
Additionally, voluntary study participation will be emphasized throughout the project, and participants will be given the option to decline participation or withdraw from the study at any time, without giving a reason.

Study design and participant burden:
The study design and procedures have been thoroughly justified to ensure that they are reasonable and not overly burdensome for participants. Participants are required to take monthly online questionnaires, two saliva tests and three visits to a skin test center. Participants will be informed of the time and effort required for participation and will be compensated appropriately. At the end of the study they receive all their personal results (if agreed to), for which the tests have a value of approximately 1000 CHF.

Overall fair balance:
In summary, the study design and procedures have been justified, and project-specific ethical aspects have been taken into consideration. Participants will be given the opportunity to make an informed decision about their participation in the study, and their rights and welfare will be protected. The burden and time effort required of participants will be minimized, and they will be compensated for their participation. Overall, there is a fair balance for the study participants.

### 6.2 Risk-Benefit Assessment

The benefits for participants of the projects are the following:
Participants in this study stand to benefit by receiving information about their biological age and their skin quality, which has the potential to improve their welfare and health awareness. These benefits are not only valuable to participants in the short term, but could also have important long-term health implications. By providing participants with free valuable health information, the study team is helping to promote participant welfare and ensure that the project is conducted in an ethical and responsible manner.

There are only minimal risks associated with the intervention participants are exposed to and procedures included in this project. They are addressed in the following section together with the risk minimization:

Avea collagen activator (exposure)

- As of now there are no known adverse reactions associated with the product from any of the early trials and since the product has been on the market, there have been no reports of major adverse effects or reactions.

Skin analysis (Corneometer, cutometer, ultrascan (22Mhz), Visia scan

- Risk minimisation during the recruitment process through screening for any sensitivity regarding exposure to light (sunlight, UV-light etc.)
- A medical professional is accompanying the trial participant during that process and on the lookout for any adverse reactions or complications during the process
- All procedures that are part of the skin analysis are non-invasive. In rare cases they can lead to skin irritation or damage, specifically if they are used too often or with too much pressure, which our study personal will be instructed to avoid.
- All skin tests will not be performed on open wounds or skin infections.

Saliva collection

- The risks are minimized as the sample collection is non-invasive (participant spits on a clean spoon).
- The sample collection is self-administered and participants will be provided with clear and concise instructions on how to collect their saliva sample.

Analysis of saliva samples to determine biological age

- The saliva samples are collectively shipped every week (the samples that are ready) to our collaborator, Trume labs, without risk to the samples as they are stable up to one year under ambient conditions.
- For the analysis, 9 small DNA regions are sequenced, and used to determine the methylation of your DNA. Genetic information is not analyzed.
- After the determination of biological age, the sequence data is deleted and the saliva samples are destroyed. This minimizes the risks of the data being used in any other way or of personal genetic information being revealed.
- The samples are screened upon arrival at Trume labs and in the case that a sample is contaminated or too wet, we will be informed and ask the participant to retake the saliva sample. As we ship the samples every week, in the case that a sample is contaminated it will be possible for the participant to redo the sample within 1 to 1.5 months maximum considering shipping times. The delay of the datapoint will be taken into account for the analysis.
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Questionnaire

- The private information provided in the questionnaires will be kept confidential, following our protocol for data protection
- Contact details of trial doctor's are provided to the patients for any case of privacy concerns, mental support or language difficulties

### 6.3 Rationale for the inclusion of vulnerable participants

This project will not be conducted in a vulnerable population.

## 7. Quality CONTROL AND Data protection

### 7.1 Quality measures

For quality assurance the Ethics Committee may visit the research sites. Direct access to the source data and all project related files and documents will be granted on such occasions.

Double data entry: Where possible, data will be entered twice by two different individuals to ensure accuracy and completeness. Any discrepancies between the two entries can be identified and resolved.
Project personnel training: Project personnel will be trained on all important project-related aspects, including data collection procedures, ethical guidelines, and quality control procedures. This will help ensure consistency and standardization across the project.
Planned quality visits: Planned quality visits will be scheduled to monitor the project's progress and identify any potential issues or areas for improvement. This will help ensure that the project is progressing according to plan and that data quality is maintained.
Independent data review: An independent data review can be conducted by an external expert or consultant to ensure that the data collected and analyzed is accurate and of high quality. This can provide an objective assessment of the project's strengths and weaknesses.
Data quality checks: Data quality checks will be conducted periodically to ensure that the data collected is accurate and complete. This will involve checking for missing data, outliers, and data inconsistencies.

### 7.2 Data recording and source data

All the study data will be recorded via an electronic Case Report Form (eCRF) in Redcap. Data quality checks will be conducted periodically to ensure that the data collected in the eCRF is accurate and complete. This will involve checking for missing data, outliers, and data inconsistencies. To ensure data traceability, we will implement an audit trail to track any changes made to the data and who made them. Any discrepancies or errors will be documented and investigated immediately. Additionally, all research personnel involved in data collection, storage, and analysis will receive training on data protection and confidentiality.

Where possible, data is directly entered into redcap, also the questionnaires performed by the participants. At the clinic, there is the possibility that the readouts are first safed in the internal systems and then transferred to redcap, these records will be stored in a secure place at the clinic.

### 7.3 Confidentiality and coding

**Project data** will be handled with uttermost discretion and is only accessible to authorized personnel who require the data to fulfill their duties within the scope of the research project. On the CRFs and other project specific documents, participants are only identified by a unique participant number.

For our study, we will be using coded non-genetic data, as well as biological material that will be analyzed for genetic data and will also be coded. The participant identification list will be stored separately from the research data by Hautwerk, and will only be accessible to authorized personnel with specific permissions. All data will be stored on secure servers and protected by passwords, encryption, and other security measures to prevent unauthorized access, alteration, deletion, copying, and theft. We will also have safety backups of all data on secure storage media to ensure that data is not lost.
To ensure traceability, we will implement an audit trail to track any changes made to the data and who made them. Any discrepancies or errors will be documented and investigated immediately. Additionally, all research personnel involved in data collection, storage, and analysis will receive training on data protection and confidentiality.

**Biological material** in this project is not identified by participant name but by a unique participant number (code). Biological material is appropriately stored in a restricted area only

accessible to authorized personnel. All biological samples are analyzed by TruMe labs. The encrypting of the saliva samples is already performed at the Hautwerk clinic; specifically, the code of each saliva sample kit (6 digits) is noted down for the participant using the specific kit by the study doctor in the case report from. The rest of the study team will then only see the unique participant number and the allocated code of the saliva sample kit. For the analysis by TruMe laboratories, only the unique participant number will be used to identify whom the sample belongs to. Therefore, it is not possible for TruMe laboratories to identify a participant and they do not receive any identifying information apart from the unique participant number.

Measures taken to prevent unauthorized or accidental disclosure and to prevent the biological material from being altered, destroyed, or stolen at TruMe Laboratories:

- Upon receiving the sample, it undergoes a visual inspection to ensure it is not contaminated or too wet.
- If the sample passes the visual inspection, DNA extraction and bisulfite conversion are performed to differentiate the methylated and unmethylated DNA.
- Small regions of DNA are extracted and sequenced to quantify a percentage of DNA methylation.
- Independent laboratory services are used to perform all sequencing procedures.
- Personally identifiable information is not provided to TruMe, they only receive encrypted information to ensure confidentiality.
- Multiple layers of encryption are used to protect the data.
- TruMe never provides your information to your employer or health insurance company.
- Individual-level information is not shared with any third party without explicit consent.
- Information is only provided to law enforcement if required to comply with a valid subpoena or court-ordered request.

Processes in place to ensure traceability of biological material:

- Upon receiving the sample, it is scanned into an internal registration system.
- The laboratory sends the resulting data to TruMe along with the barcode that came with the sample.
- TruMe does not receive personal identifiable information for participants of this study.

Storage and technical requirements to be met:

- The laboratory maintains a cooling system to ensure appropriate storage of biological material.

Shipping biological material or data collected during the research project outside the research site:

- the receiver address: TruMe, Inc., 1115 Holly Street Alameda, CA 94502, US
- responsible person to whom materials or data are sent: Yelena Budovskaya
- purpose of shipment: Analysis of saliva samples to get biological age
- temperature control: not necessary, stable at ambient temperatures
- participant confidentiality is guaranteed via separating Personally identifiable information (PII) and personal health information (PHI).
- Participants are being sufficiently informed and consent is obtained that their biological material is being sent to the US to the contracted laboratory (Trume labs) that performes the analysis of the saliva samples.

The decision to conduct these analyses in the USA is rooted in a combination of scientific precedent and practical considerations. In a previous study[21] that utilized the same company and testing method for analyzing biological age, remarkable outcomes were observed. This study indicated an average reduction of 8 years in biological age after an average of 7 months, and notably, the supplement used also contained alpha-ketoglutarate. Given this promising precedent, we aim to replicate these impressive results in our current study, maintaining consistency with the methods that led to such a meaningful outcome previously.

Furthermore, we've also considered the availability of alternative options within Switzerland. While ensuring local analysis is desirable, unfortunately, there are no local competitors who offer similar analyses at a cost comparable to the arrangement we have with our current US-based partner. Budget constraints are a significant consideration for our study, and partnering with the US-based company allows us to achieve comprehensive and high-quality analyses without compromising the study's financial scope.

Swiss dataprotection regulations upheld in the US:

- All saliva samples sent to the US laboratory are encoded using encryption protocols, which adhere to both Swiss Federal Data Protection Act (FADP) and US data security standards. This step ensures that the genetic material remains secure and unreadable during transmission and analysis, minimizing the risk of unauthorized access.
- As the scope of analysis is limited to only 9 short gene sequences directly relevant to determining biological age, this focused approach ensures that only the necessary genetic information is utilized, in alignment with Swiss data protection principles of data minimization. Once this analysis is completed and the biological age is determined, the genetic information used for this specific purpose is promptly deleted. This precautionary measure adheres to both Swiss and US regulations and minimizes the retention of sensitive information to what is strictly essential.
- Throughout the entire process, the US laboratory conducting the analysis does not have access to any identifying information linked to the samples. This includes personal details of the participants or any contextual information. The encrypted data ensures that the laboratory lacks the capacity to link the samples to specific individuals, thereby preserving the anonymity of participants.
- Further, strict access controls are in place at TruMe laboratories. Only authorized personnel directly involved in the analysis process have limited access to the encrypted genetic data. This controlled access ensures that information is handled responsibly and with the utmost care.
- From each participant informed consent will be obtained and they are informed about the details of the analysis and that it is taking place in the US. This transparent communication ensures that participants are aware of the process and can make an informed decision about their participation, in alignment with Swiss data protection guidelines. Further, they are informed that withdrawal of the study is possible at any time and without reason.

### 7.4 Retention and destruction of project data and biological material

The health-related data collected during the study will be encrypted and access controlled stored for a duration of 10 years after the publication of the research project by the Sponsor in RedCap. Whereas, the participant key list will be encrypted and stored for as well 10 years after the publication of the research project by Hautwerk. After the completion of the study, biological materials will be destroyed and will not be used for further analysis or a biobank. The destruction of biological materials will be documented in accordance with the ethical guidelines. If applicable, the data may be used further, for example, in meta-analyses, systematic reviews, or for further research in related fields. Any additional use of the data beyond the original research purpose will comply with ethical guidelines and require a new ethical review process.

## 8 Funding / Publication / declaration of Interest

The project is industry funded through the sponsor (Avea life AG). Publication of the study's results will be conducted jointly with Avea life AG and Collin Ewald, assistant professor at ETH, who was involved in the development of the product being tested.
As a result of the collaborative development of the product, there may be potential conflicts of interest. However, to ensure transparency, all relevant disclosures will be made in the publication.
In terms of data sharing, we plan to make the data available in a publicly accessible repository after the study has been completed. Any specific requirements for data sharing or limitations on the use of the data will be detailed in the publication and/or in a separate data sharing agreement.
This information is also captured in the contract between Avea Life AG and the Principal Investigator, and in any other relevant agreements or documents related to this study.

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## Appendix 1: Schedule of assessments

| | ***>-1 day*** | ***-1*** | ***0*** | ***Month 1*** | ***Month 2*** | ***Month 3*** | ***Month 4 + 5*** | ***Month 6*** |
|---|---|---|---|---|---|---|---|---|
| ***Visit*** | *Information* | *Screening (online)* | *1st visit* | *2nd visit* | *Online check-in* | *3nd visit* | *Online check-in* | *Online check-in* |
| ***Information (written)*** | + | | | | | | | |
| ***check inclusion-/exclusion criteria*** | | + | + | | | | | |
| ***Participant characteristics*** | | + | | | | | | |
| ***Written consent*** | | | + | | | | | |
| ***Skin Tests*** | | | + | + | | + | | |
| ***Questionnaire*** | | | + | + | + | + | + | + |
| ***Saliva sample*** | | | + | | | | | + |