Test plan No. 2017-2

Effects on the bioavailability of β-carotene when taken concomitantly with zinc and iron supplements

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List of abbreviations

ADR Adverse Drug Reaction
AE Adverse Event
AUC Area-under-the-curve
BMI Body Mass Index
FDA Food and Drug Administration, U.S. Food and Drug Administration
GCP Guidance on Good Clinical Practice
HPLC High-pressure liquid chromatography
RDA Recommended Daily Allowances
SAE Serious Adverse Event
SOP Standard Operating Procedure
UE Adverse Event
Abstract of the project

Title:
Effects on the bioavailability of β-carotene when taken concomitantly with zinc and iron supplements.
1. study objective
The objective of this study is to investigate the effect of concurrent intake of zinc or iron supplements on the bioavailability of β-carotene. The proposed trial is a purely scientific controlled, randomized, double-blind, prospective nutritional study.

2. scientific background
Iron, zinc, and vitamin A deficiencies are global public health problems and particularly affect pregnant and lactating women and children, who are especially vulnerable during the so-called 1000-day window, the period from conception to the second birthday. According to the Global Nutrition Report (2015), 29% of women of childbearing age worldwide are anemic due to iron deficiency, 15% of newborns have low birth weight, and 164 million children under the age of five are developmentally inhibited, which are signs of zinc deficiency. The situation is worse in developing countries, where an estimated 50% of pregnant women and 40% of preschool children are anemic (WHO, 2016). A recent summary analysis showed an increase in the prevalence of vitamin A deficiency in sub-Saharan populations from 45% in 1991 to 48% in 2013 (Stevens et al., 2015). These deficiencies are often treated in vulnerable populations through administration of supplements, increased dietary intake, and dietary modification. While vitamin A deficiency is increasingly treated by switching to a diet rich in β-carotene (provitamin A), severe zinc and iron deficiencies are still largely treated with supplements. Taking zinc supplements can greatly reduce the frequency and incidence of diarrhea in children under five years of age (IOM, 2001), and iron supplementation is still the preferred treatment for iron deficiency anemia (IDA) (WHO, 1998).

Since there is usually a simultaneous deficiency of vitamin A, iron and zinc, a combination of these micronutrient supplements is often recommended. However, in vitro studies suggest that intestinal absorption of β-carotene may be inhibited by iron and zinc (Bengtsson et al., 2009; Biehler et al., 2011). Fat-soluble β-carotene, in order to be absorbed in the small intestine, must be incorporated into so-called mixed micelles under the action of bile salts. Caco-2 cells incubated with micellated β-carotene and different concentrations of iron absorbed on average

22% less β-carotene in the presence of iron (Bengtsson et al., 2009). Another study examined the effects of different concentrations of magnesium, calcium, iron, and zinc on the ability to form micelles and uptake β-carotene in Caco-2 cells (Biehler et al., 2011). The micellation of β-carotene and its uptake into Caco-2 cells decreased with increasing concentrations of the minerals. Iron and zinc had a much stronger inhibitory effect than calcium and magnesium. In both studies, the authors attributed the decreased absorption of β-carotene to an interaction of iron with bile salts and a resulting decreased micellation.

Experiments performed in our laboratory with Caco-2 cells confirm the results of the above studies, as we also measured a dose-dependent reduction in micellation of β-carotene with increasing iron concentration.

To the best of our knowledge, no human studies have been performed to determine the influence of iron and zinc supplements on the bioavailability of β-carotene. However, these are of great importance and relevance because in vitro systems are not dynamic and cannot adequately model the influence of minerals on β-carotene bioavailability. For example, it is possible that the continuous secretion of bile salts in vivo may compensate for precipitation by minerals and thus the reduction in absorption.

3. Hypothesis

Based on the in vitro experiments described above (see 2), which observed reduced micellization of β-carotene in the presence of iron and zinc, we will test the hypothesis that when β-carotene is taken simultaneously with iron or zinc, its bioavailability is reduced in healthy subjects.

3.1 Randomization and Blinding

To avoid systematic errors and otherwise influencing the results, the study will be randomized and double-blinded. Randomization and blinding will be done before the start of the study and will be performed by a staff member who is not actively involved in the study. For this purpose, the samples obtained will be randomly numbered and the key to unblinding, i.e., the assignment of the randomly generated sample numbers to the specific samples (identified by time of sampling and type of intervention), will be stored in sealed envelopes in two different locations in locked cabinets.

Subjects receive the different preparations (15 mg β-carotene and placebo, 15 mg β-carotene with 25 mg iron, or 15 mg β-carotene with 30 mg zinc) in capsules in random order. Analysis of plasma samples was also randomized and blinded to prevent inadvertent influence of the experimenter on the results. Unblinding of the samples for statistical analysis of the data is done only after all biochemical analyses have been completed.

3.1 Target parameters and evaluation

The bioavailability of β-carotene is determined via the measured plasma and chylomicron concentrations by HPLC (see 8.6 Methods). For this purpose, ultracentrifugation is used to isolate the chylomicron fraction and extract β-carotene. Pharmacokinetics for plasma and chylomicron fraction are calculated using the area under the concentration-time curve. This quantity can be used to quantify the bioavailability of β-carotene in comparison between single and combined intakes. Statistical analysis is performed using one-factor analysis of variance.

4. study population

4.1 Number of subjects

The required power for the study will be calculated based on the area under the plasma concentration-time curve (AUC). Study power is calculated based on the following formula: n > F (σ/d)2, where n = number of subjects, σ = standard deviation of the dependent primary outcome measure, d = difference between control and treatment classified as significantly different in the literature. The corresponding F-values can be found in the table below; for the planned study, P < 0.05 and 90% power are assumed, resulting in an F-value of 10.51 (Scales & Rubenfeld 2005).

F-value Table:

	Power			
Significance level (P-value)	80%	**90%**	95%	99%
0.100	6,18	8,56	10,82	15,77
0.050	7,85	**10,51**	12,99	18,37
0.025	9,51	12,41	15,10	20,86
0.010	11,68	14,88	17,81	24,03

In a comparable study by (Goltz et al. 2013), an AUC of 22.6 ± 7.6 [nmol*L-1*h] could be determined after intake of 20-30 mg β-carotene (n=6). This results in a mean value of 22.6 nmol*L-1*h and a standard deviation of 7.6 nmol*L-1*h. A reduction of bioavailability by more than one third would represent a biologically relevant effect, which is why a significant difference of rounded up 35%, corresponding to 7.9 nmol*L-1*h, is used for the calculation in this study. Thus, n > 10.51 x (7.6/7.9)2 results in a number of at least 9.7, rounded up to 10 subjects. To allow for a balanced experimental design in which all permutations of the timing of the interventions occur in equal numbers, a total of 12 healthy males aged 18-45 years will be recruited for this study. Females will be excluded from the study because the menstrual cycle affects blood concentrations of carotenoids (Forman et al. 1998).

4.2 Duration of the study

The duration of the study is approximately 3 weeks per subject and includes 3 interventions, each preceded by a 1-week washout period. The three interventions each consist of a single oral administration of 15 mg β-carotene with placebo, in combination with 25 mg iron (FeSO4), or in combination with 30 mg zinc (ZnSO4).

5. selection of subjects

5.1 Inclusion criteria

Healthy, consenting males aged 18 to 45 years are sought for the study. A prerequisite for participation is that the small blood count, parameters of liver and kidney function, lipid metabolism, inflammatory markers, blood pressure and body mass index (BMI) are within the normal range.

5.2 Exclusion criteria

Subjects will not be included in the study should at least one of the following exclusion criteria be present: Use of medication, previous myocardial infarction, addiction, malignant disease, diabetes mellitus, dementia, arterial hypertension, smoking, increased alcohol consumption, use of multivitamin tablets or food supplements, extreme physical stress (>5 hours of exercise

per week) and in case of a known intolerance/hypersensitivity to the preparation (β-carotene, iron, zinc or fillers used).
Subjects enrolled in another study during the same time period or who have participated in another clinical trial within the previous 3 weeks will not be enrolled in the study.

5.3 Mode of recruitment
Subjects will be recruited by means of posters, flyers, requests on the websites of the participating study groups and institutes, and advertisements in local daily newspapers. During the initial telephone interview, a short interview is conducted to verify that the subjects meet the inclusion criteria of the study. When a sufficiently large number of potential participants has been gathered, "screening" takes place. Study participants will receive an expense allowance of EUR 250.00 at the end of the study.

5.4 Informed consent of study participants
A subject can only be included in the study if he/she has given his/her consent to this after being informed orally and in writing by an investigator about the nature, significance and scope of the study in an appropriate and comprehensible manner.
At the same time as giving his consent, he must have declared that he agrees to the recording of data within the framework of the study and to their review by authorized persons (e.g. monitor, auditor). It must be clear to him that he can withdraw his consent at any time and without giving reasons, without any disadvantages resulting from this.
The original of the written consent will be kept in the study folder of the study center. The subject will be given a copy of the written patient information and the informed consent form. In addition, copies of both documents will be filed in the study folder. Patient information, consent form and all other documents received by the participants as well as recruitment notifications will be submitted to the responsible ethics committee for approval before use. See informed consent forms for both the screening and participation attached at the end of the document.

6. Test medication

6.1 Pharmacological-toxicological expert opinion
Since vitamin A (retinol) can be formed from β-carotene, including in the intestine and liver, it enters the reference values for the desirable daily intake of vitamin A (6 mg β-carotene corresponds to 1 mg retinol). β-Carotene is part of the daily diet and is absorbed mainly with intensely colored vegetables and fruits (spinach, kale, broccoli, lettuce, carrots, tomatoes, pumpkin, mango, etc.). Individuals with a high fruit and vegetable intake (5 servings per day) consume a mean of 36 mg of β-carotene per day (Salter-Venzon et al. 2016). Because βcarotene contributes to vitamin A supply but is not itself an essential nutrient, no adverse health effects are expected even with several days of abstinence. In the CARET (Carotene and Retinol Efficacy Trial) study, long-term intake (4 years on average) of 30 mg daily of β-carotene and 25,000 IU retinol (vitamin A) increased the risk of lung cancer in smokers and persons with asbestos exposure, but not in nonsmokers (Omenn et al., 1996). However, no increased risk is expected with a single intake of β-carotene alone. Nevertheless, to exclude any risk, only nonsmokers are included in the study.

The German Nutrition Society (DGE) recommends a daily intake of 10 mg iron for adult men. Adults with normal intestinal function have only a very low risk of being exposed to too much dietary iron (NIH, 2016). However, ingestion of more than 20 mg/kg body weight (equivalent to 1.4 g for an average adult of 70 kg body weight) from dietary supplements or medical

preparations can cause stomach upset, nausea, constipation, abdominal pain, vomiting, and feelings of weakness, especially if taken fasting. Sustained use of iron supplements containing 25 mg elemental iron or more may result in decreased absorption of zinc and decreased zinc plasma levels. In severe cases (for example, a single dose of 60 mg/kg), iron overdose can lead to multiple organ failure, fainting, coma, convulsions, and even death ((Papanikolaou & Pantopoulos 2005). In the case of a single intake of 25 mg iron (equivalent to the iron content of approx. 100 g blood sausage or pork liver), as planned in the present study, no health risks are to be expected for the subjects.

The daily intake of zinc recommended by the German Nutrition Society (DGE) for adult men is 10 mg. The toxicity threshold for zinc is very high. Acute poisoning with 2 g of zinc causes gastrointestinal disturbances and fever. Acute poisoning from very high doses of zinc manifests primarily as nausea, abdominal cramps, vomiting, tenesmus, and diarrhea (possibly bloody), but is very rare (Maret & Sandstead, 2006). One case study reported severe nausea and vomiting within 30 min of ingesting 4 g of zinc gluconate (equivalent to 570 mg of elemental zinc; NIH, 2016). Daily intakes of 150-450 mg of zinc are associated with low copper status, changes in iron function, lowered immune system activity, and reduced HDL concentrations. Daily intake of 60 mg zinc for 10 weeks resulted in decreased copper-containing enzymes, which are a marker of copper status. The AREDS study with a daily dose of 80 mg zinc in the form of zinc oxide for an average of 6.3 years showed increased hospitalizations for urogenital complaints. This suggests that chronic intake of zinc has effects on certain aspects in urinary tract physiology. Taking 30 mg of zinc once with a commercially available dietary supplement, as planned in the present study, does not pose a risk of adverse health effects to the subjects.

In this study, only commercially available low-dose dietary supplements are used and administered in single doses. Therefore, there are no health risks associated with either the administration of the micronutrients or the one-week washout periods with a low-carotenoid diet for the subjects.

7. medication

7.1 Dosage and mode of administration

Subjects (n=12) will receive, in random order, a single oral dose of 15 mg β-carotene individually plus a placebo capsule (filled with silica and maltodextrin), 15 mg β-carotene plus 25 mg iron ($FeSO_4$), or 15 mg β-carotene plus 30 mg zinc ($ZnSO_4$) in a double-blinded crossover study (interventions separated by one-week washout periods). Commercially available dietary supplements are used for this purpose (β-carotene from BIOVEA, Ferrogamma (iron) and Zinkit® (zinc) from Wörwag Pharma GmbH & Co. KG).

8. study procedure

8.1 Screening

Subjects are considered to be enrolled in the study if they have given their written informed consent to participate in the study (see section 5.4).

First, it is checked that all inclusion criteria are fulfilled and that none of the exclusion criteria are present. For this purpose, a medical history is taken regarding the subjects' health status and dietary and exercise habits. The subjects are weighed and measured. Furthermore, a blood sample will be taken by medical staff (investigator: Dr. Dr. Venturelli). A small blood count will be collected from each subject.

8.2 Hospitalization
The screening of the subjects and the study will take place at the Institute of Biological Chemistry and Nutrition Science. Hospitalization of the subjects is not required.

8.3 Nutrition
Standardized meals will be served on the study days (from the evening before the intervention until the collection of the 10 h blood sample). Subjects will be instructed not to consume foods containing β-carotene (see Subject Information and Supplementary Information) during the studies. An appropriate list of foods and beverages to be avoided will be given to subjects as part of the subject information.

The standardized meals are composed as follows:
Dinner before the first study day consists of farmhouse bread with butter and camembert, plus roasted macadamia nuts and natural yogurt without artificial colors or additives. For breakfast on the study day, fresh wheat rolls with cream cheese (natural) and Greek yogurt are served in parallel with taking the capsules. Lunch on the study day is served four hours after taking the capsules and consists of pasta with cheese sauce, rolls and yogurt ice cream for dessert. The snack 6 hours after intervention consists of ricotta cake. For dinner on the study day, potato salad, farmhouse bread with butter, almonds, and a yogurt are served. All foods used were chosen from the point of view of avoiding additional intake of β-carotene.

8.4 Documentation
Each subject is assigned an ID number and all study data (which preparation the subject received, blood test findings) are recorded under the respective ID number.

8.5 Experimental procedure
Subjects are considered to be enrolled in the study if all inclusion criteria have been met during screening (see section 5.1), none of the exclusion criteria (see section 5.2) are present and the subjects have given their written consent to participate in the study.

On the evening before the intervention, subjects will receive a standardized meal and a standardized snack for consumption between dinner and 10 pm. Subjects will be asked to drink only water and consume no other food after 10 pm and until the intervention on the day of the trial. On the morning of the trial day, subjects will receive a single oral dose of 15 mg β-carotene plus placebo, 15 mg β-carotene combined with 25 mg iron ($FeSO_4$), or 15 mg β-carotene combined with 30 mg zinc ($ZnSO_4$) after breakfast. Standardized meals will be served to study participants throughout the day of the intervention. Blood samples will be collected at baseline (0 h) and 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h after ingestion of the study drugs (11 blood samples). A total of approximately 120 mL of blood will be collected per intervention.

As the study is a cross-over study with three arms, after each 1-week washout period, the same experimental procedure is performed in random order with one of the remaining study preparations each time.

8.6 Methods
The blood test (for screening) is performed by the joint practice for laboratory medicine "Laborärzte Sindelfingen" (Nüßstraße 5, 71065 Sindelfingen, phone 07031-79930).

For the investigation of bioavailability, β-carotene is measured in the blood by means of highpressure liquid chromatography (HPLC), which were taken over the entire period. The appropriate HPLC analysis for this purpose has been established (Stuetz et al 2016). Isolation of the chylomicron fraction is performed according to (Goltz et al 2012).

9. precautionary measures / discontinuation criteria

9.1 Risk-Benefit Consideration.

There is no direct benefit to the subjects of the study. They will receive the result of their blood values, as well as meals on the day of the trial and the evening before. Should indications of health problems of a potential subject come to light in the course of the study or screening, the subject in question will be informed of this by the investigator and, if necessary, advised to consult a specific specialist. The risks of blood collection are a short-term uncomfortable sensation from the needle prick, rarely bruising, and very rarely infection. Participating routine physicians will perform the blood draws so that complications or risks to study participants can be minimized and virtually eliminated.

9.2 Adverse events

Since subjects in the planned study will consume only a single oral dose each of 25 mg iron ($FeSO_4$) and 30 mg zinc ($ZnSO_4$), and only three times a dose of 15 mg β-carotene, no health risks to subjects are expected. As described in 6.1, side effects appeared only with long-term intake of very high doses. Furthermore, in this study only commercially available supplements in the form of capsules are administered.

Nevertheless, the principal investigator agrees to document all UE or Adverse Events (AE) as well as side effects of the supplements or Adverse Drug Reactions (ADR) that might occur during the course of the studies. When AEs or ADRs occur, information on the type, time of occurrence, and duration as well as intensity and frequency of the adverse event will be documented. AE or ADR are defined as events such as mild nausea, mild dizziness, mild reversible change in blood count.

Furthermore, the measure taken as well as a connection with the intake of the preparation or whether the symptom was already known before the start of the study is noted. If the event is related to the study conduct, the investigator commits to immediately exclude the subject from the study.

9.3 Serious Adverse Events and Serious Unexpected Side Effects

Serious adverse events are defined as events that may lead, for example, to hospitalization, persistent disability, life-threatening impairment or even death.

Suspected Unexpected Adverse Reaction (SUSAR) is an adverse event that is inconsistent in nature or severity with the available information on the investigational product, is judged to be serious, and for which a relationship to the investigational drug is judged to be at least possible by the investigator.

9.4 Discontinuation criteria

9.4.1 Discontinuation of the study in one subject (drop-out).

The study is discontinued in an individual subject if the subject withdraws consent to participate in the study, the study protocol is violated, an exclusion criterion or disease occurs, or other circumstances arise that would jeopardize the subject's health if the subject continued to participate in the study.

9.4.2 Termination of the entire study

If the suspected case of a serious unexpected adverse reaction occurs during the human study after taking 15 mg β-carotene plus placebo, 15 mg β-carotene with 25 mg iron (FeSO4), and 15 mg β-carotene with 30 mg zinc (ZnSO4), the study will be terminated at any time for the benefit and in the interest of the subjects. In this case, the Ethics Committee would be informed immediately.

10 Data collection and analysis

Statistical analysis will be performed by the study coordinator. Bioavailability will be determined by the area under the curve (AUC), maximum plasma concentration (Cmax), and time to reach Cmax (Tmax) of β-carotene. Significant differences between interventions will be determined using GraphPad Prism 6.0 software using one-factorial analysis of variance for repeated measures.

11. amendments to the protocol.

The applicants are aware that the vote of the Ethics Committee covers only the information contained in the application and does not include extensions and changes to the research proposal made at a later date. The applicants are aware that in case of changes, a protocol annex is required, which must be signed by the study directors, and that all protocol changes must be reported to the Ethics Committee. The applicants are aware that a new vote of the Ethics Committee must be obtained for protocol changes that are not exclusively formal in nature and that contain changes relevant to the study participants. Potential changes in study conditions would be brought to the attention of study participants during the informed consent process.

12 Ethical and legal issues

12.1 Legal basis

The study will be conducted in accordance with the Declaration of Helsinki (as amended) and the Guidance on Good Clinical Practice (GCP) Regulations.

12.2 Vote of the Ethics Committee

The applicants are aware that the prerequisite of the nutritional study is compliance with the guidelines and laws listed under item 12.1 and that the initiation and conduct of the study are subject to the vote of the Ethics Committee. The Ethics Committee will be informed immediately in the event of serious events (section 9.3), study termination (section 9.4) and protocol changes (section 11). After completion of the study, the Ethics Committee will be provided with a report and, if necessary, a publication.

12.3 Documentation, archiving and data protection

The recording of the collected findings is done by means of a test sheet. Among other things, the test sheet will summarize information about the participating person in pseudonymized form (ID number), information about which 15 mg β-carotene with placebo capsule, 15 mg βcarotene with 25 mg iron (FeSO4) and 15 mg β-carotene with 30 mg zinc (ZnSO4) preparation the person received, findings of the blood test, etc.

All data collected in the study will be subject to data protection and will be kept strictly confidential. Samples will be encrypted and no report or publication will reveal the identity of study participants. Blood samples will leave the facility in encrypted form. The transfer of test forms and data storage for evaluation will only be done with the anonymized data of the study participant. Assignment of personal data to study data will be made only by the study director. After completion of the study, the data will be stored for a period of 10 years.

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Dear Participant,

Thank you for taking part in the screening for the study **"The effect of simultaneous oral administration of zinc and iron supplements on the bioavailability of β-carotene"**.

Our study doctor, will now take two tubes of blood (10 ml each) for the determination of the following parameters:

- Bilirubin (total)
- Uric acid
- Triglycerides
- Creatinine
- Alkaline phosphotase
- Glutamate oxaloacetate transaminase
- Gamma-glutamyl transferase
- Glutamate pyruvate transferase
- Total cholesterol
- HDL cholesterol
- LDL cholesterol
- Blood count

In the next step, we will determine your body mass index (height and weight) and your blood pressure. The information collected will be kept confidential, but we will inform your laboratory (Laborer Sindelfingen), who will perform the blood tests for us, your name and date of birth for the correct assignment of the samples.
You will not be compensated for the screening. However, you are invited to a small breakfast after the screening. In addition, you will receive the results of the blood tests by email.
We will contact you as soon as possible and inform you about the further course of the study (the participant information you have already received by e-mail).

Consent
I hereby agree to participate in the screening for the study "Effects on the bioavailability of β-carotene during oral administration of zinc and iron preparations" comprising the above-described tests.

_____ _____
(Place, Date) (Participant signature)

_____ _____
(Place, Date) (Dr. Dr. Sascha Venturelli)

Version 4, 2. Oktober 2017

Participant information

(October 2017)

"The effect of simultaneous oral administration of zinc and iron supplements on the bioavailability of β-carotene"

In the event of any ambiguities, emergencies, unexpected or undesirable occurrences during this study, please contact the following contact person at any time:

Universität Hohenheim
Institut für Biologische Chemie und Ernährungswissenschaft
Fg. Biofunktionalität und Sicherheit der Lebensmittel (140b)
Jan Frank
Garbenstraße 28
70599 Stuttgart
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Email: carotinstudie@nutres.de

Version 4, 2. Oktober 2017

Dear participant,

please read this leaflet carefully. You will be given sufficient time and opportunity to clarify all questions related to your participation. If you have any further questions or comments, please do not hesitate to contact us. After the information you will be asked to sign an agreement to participate in the study. With this declaration of consent, you confirm that you have been informed in detail about the study, that your participation in the study is voluntary and that you can terminate your participation at any time.

1. What is the purpose of the study?

1. What is the purpose of this study?

The aim of this study is to investigate the influence of zinc and iron supplements on the bioavailability (intake in the intestine and blood concentrations) of β-carotene (a precursor of vitamin A). Iron, zinc and vitamin A deficiency are widespread deficiencies around the world that can be caused by sub-optimal nutrition. For prevention and treatment of nutritional deficiencies, food supplements are often administered with the appropriate nutrient. In the case of simultaneous occurrence of several deficiencies, preparations with a combination of the nutrients are also administered.

In vitro studies (experiments in an artificially produced environment outside a living organism) have shown that increased iron and zinc intake due to supplements can reduce the bioaccesibility and uptake of β-carotene from food. The current study therefore aims to investigate whether the simultaneous consmption of zinc or iron with β-carotene results in a reduced absorption and lower blood concentrations compared to β-carotene consumed alone. The prospective study is a controlled, randomized, double-blind, study of cross-over design.

2. Study preparations

Each study day the participant will be given 15 mg of β-carotene (corresponds to a capsule of the brand BIOVEA Beta Carotene) per intervention. You will also receive either 25 mg of iron (Ferrogamma from Wörwag Pharma GmbH & Co. KG), 30 mg of zinc (zincite from Wörwag Pharma GmbH & Co. KG) or capsule-type placebo. The preparations administered are food supplements, that are also available in the supermarket and in drugstores.

3. Study execution

A total of 12 healthy subjects are recruited (men aged 18-45 years). The duration of the study is approximately 3-5 weeks for each subject.

There are 3 intervention days (times of administration) which are separated by a minimum of 1-week washout phase (period in which no test substance is administered). Each intervention is

characterized by a one-time oral preparation, so that immediately after ingestion the time window of the wash-out phase begins.

The selection period is to take place in October 2017 and the study itself takes place in October/November 2017. All examinations and samples are given at the Institute of Biological Chemistry and Nutritional Science, Garbenstr. 28-30, 70599 Stuttgart under medical supervision. The following investigations are carried out after clarification and voluntary consent to the study:

Screening

Before entering the study, each candidate is subjected to a preliminary examination. This includes a medical survey, measurement of blood pressure, body weight and body size. Furthermore, routine blood tests are performed to check general health. In addition to the small blood count, the hepatic enzymes used to check the liver function, the creatinine level as a parameter for the renal function and the blood fat values are determined as routine parameters. The blood sample must be taken after at least 10 hours of dietary fasting, ie before breakfast. The time required for this is about 30 minutes. On the basis of this data, it will be decided whether the necessary prerequisites for the study participation are fulfilled.

Study day visits 1, 2 and 3

Time: approx. 12 hours with free time

After at least 10 hours of fasting, the first blood collection takes place for the determination of the baseline. For this purpose, a permanent venous approach in the arm is inserted by the study doctor. The cannula remains in your vein for approximately 10 hours for the successive blood draws. You will be given a breakfast with which the capsules are taken with β-carotene alone and placebo or in combination with iron ($FeSO_4$) or zinc ($ZnSO_4$) under supervision. The concentration course of the nutrients in the blood will be determined over a period of 10 hours. Blood samples are collected at 11 consecutive times (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 hours) after taking the capsules. After each blood sampling, the cannula is rinsed with saline solution to avoid blood clotting. The cannula is then closed until the next blood draw. Four hours after breakfast, you will receive a standardized lunch meal, after 6 hours a standardized snack and after 9 hours a dinner. It is strictly forbidden to bring or consume your own additional food and drinks, as these could change the absorption processes. You may drink water at any time without restriction.

After the 10 h blood sample has been collected on the day of the experiment, this study day is cpmpleted and you can leave the study center.

After a one-week wash-out period, the course of the study day with the other preparations is repeated in the corresponding dosage form. In order to assess the tolerability of the preparations we ask you to complete a short questionnaire, which will be handed out to you on the day of the administration.

4. General information

Nutrition

During the study period (one week before the first day of study until the end of the third day of the study) you should adjust your dietary habits. We ask you to refrain from consuming orange, yellow and green foods during the days preceding visits 1, 2 and 3 and during the wash-out phases; simply avoid colourful food and replace with white/colouless food. These limitations should only be applied to the study period (1 week before the first day of the study to the last blood test on the last day of the study) and not as a general recommendation for nutrition.

Thus, one week before and after the end of the study, β-carotene-rich foods should be avoided (see "General information on the β-carotene study") and three days before the respective study day, only the food from the list with β- Carotene-poor foods (see list at the end). On this list you will also find menu suggestions for the study period. On the evening before visits 1, 2 and 3, we ask you to consume a standardized dinner prepared by us, in order to ensure a comparable starting conditions between the individual participants and between the visits. You should also abstain from consuming alcohol 24 hours before and on the study days. In the wash-out phase we ask you to limit your alcohol consumption to a maximum of 20 g / day, which corresponds to approx. 0.2 l of wine. At study days 1, 2 and 3 you get a standard breakfast, a standard lunch meal, dinner, snacks and water. Food or drinks brought along must not be consumed on the day of the study until the final blood sampling after 10 h.

Excercise

Your usual sports activities can be maintained during the duration of the study. However, we ask you to refrain from strenuous physical activities as well as endurance sports in the 24 hours before the student days.

Medication

Intake of all medication must be documented. If the use of medication for medical reasons is necessary, please let us know.

5. What adverse events (side effects) or risks can occur?

The blood draw is done by inserting a cannula for the subsequent blood sampling corresponds to routine medical procedures. However, haematomas (bruises), infections and very rarely

inflammation of the veins, thromboses or nerve injuries can occur. Thrombosis is the occlusion of a blood vessel through a blood clot. A total of approximately 380 ml blood will be taken over the entire study including the screening (in comparison: in blood donation, 500 ml of blood is withdrawn). Please inform the physician about any adverse events occurring during the study. The doctor will, for his part, register adverse events and, if necessary, initiate necessary steps. The doctor will inform you about any new findings that may affect the benefit or safety of the study and thus your consent.

6. Protection of your personal data

The provisions on medical confidentiality and data protection are complied with in this study. During the study, medical records and personal information are collected and collected by you. The data important for the clinical examination are entered into a separate documentation sheet in a pseudonymized form (that is, encoded by the subject number and without attribution). Only these encrypted data is used. The study data are kept for 10 years.

7. Termination of the study

Your participation in this study is entirely voluntary. You can terminate your participation in the study at any time without incurring any disadvantages. It is helpful if you explain to the doctor the reason for the termination. However, you are not obliged to state reasons for your decision. Your physician may exclude you from this study in the interest of your health. If you terminate the study at an early stage, you may request the deletion of the data collected up to that point.

8. Benefits

There is no guarantee that your participation in this study will give you any personal benefit. If desired, you will receive a copy of the results of the routine laboratory parameters of your blood tests.

9. Compensation of expenses

All examinations are free; neither you nor your health insurance company has to pay anything. The study supplements and food will be made available to you free of charge. For full participation until the last day of the course, you will receive 250 €. Travel expenses are not refunded separately. In case of cancellation for personal or technical reasons, you will be compensated pro rata.

These dietary recommendations should only be observed during the study to ensure a carotene-poor diet. It is not a general dietary recommendation.

Carotene-Poor Foods

Vegetables:
Potatoes
Mushrooms
Garlic
White Onions
Celery Root
Cauliflower
White Asparagus

Fruit:
Lychee
Coconut

Starch Products:
White Noodles
Wheat Flour
White Rice
Waffles
Bread (Except Bread With Cornmeal)
Noodles

Drinks:
Coffee
Water
Wine
Beer
Low-Fat Milk

Meat / Protein-Rich Foods:
Pork Meat
Beef
Chicken
Sausage
Beef Extract
Soy Products
White Beans

Other:
Sunflower Oil
Olive Oil
Nuts (Other Than Pistachios)
Low-Fat Milk And Milk Products
Soy Milk
Coconut Milk
Almond Milk
Low-Fat Natural Yoghurt
Low Fat Fresh Cheese
Low-Fat White Cheese (Feta Cheese, Sheep's Cheese, Camembert)
Mayonnaise
Pickled White Onions
Peanut Butter
Chocolate Spread
Honey
Syrup
Salt
White Pepper
Black Pepper

Snacks:
Chocolate
Nuts
Potato Chips, Salted

<u>Use the following foods very sparingly:</u>
Colored Spices
Mustard

Examples of possible meals:

Breakfast:

Toast with cottage cheese and low-fat natural yoghurt
Pancakes with honey
Lychees with natural yoghurt
Oatmeal with low-fat milk and honey
Muesli with low-fat milk

Lunch:

Fleischkäse with potato salad
Potato salad and sausages
Mushroom soup with white bread
Chicken mayonnaise sandwich (white bread) with fries
Fleischkäse sandwich
Ham and cheese sandwiches (white bread)
Flammkuchen with onions and bacon
Sandwich with cottage cheese (white bread)
Beef burgers (without salad and tomato) with feta and bacon and French fries / potato chips
Schnitzel with rice and mushroom sauce
Chicken Nuggets
Fish sticks

Dinner:

White asparagus with ham and butter (no hollandaise sauce)
Rice with chicken and garlic sauce
Mashed potatoes with pork sausage and onion
Spätzle and pork cutlets
Grilled beef strips with cauliflower and white sauce
Ricotta stuffed noodles in mushroom sauce
Fried potatoes or salt potatoes and grilled chicken
Mushroom risotto
White bean, mushroom and chicken soup
Deep-fried / baked camembert (without cranberries)

snacks:

Nuts
Lychees
Potato Chips

Version 4, 2. Oktober 2017

Written declaration of consent to the study

"The effect of simultaneous oral administration of zinc and iron supplements on the bioavailability of β-carotene"

Place of study: Stuttgart, Germany (monocentric)
Investigator: Dr. med. Gunther Adler

Proband: _____
(Name, Surname)

I was informed verbally and in writing about the aims and the course of the study as well as about possible advantages and disadvantages as well as about possible risks. I have received, read and understood the written subject information, which belongs to the above study. My questions concerning the participation in this study or my questions concerning the medical terms used in the subject information have been answered satisfactorily. I can keep the written subject information and receive a copy of my written declaration of consent. I had enough time to make my decision. I participate voluntarily in the study. I can revoke my consent to the participation at any time and without giving reasons, without incurring any disadvantages. I am aware that during the study, the requirements and limitations stated in the subject information must be observed. In the interest of my health, the study physician can always exclude me from the study. In addition, I inform the doctor about the concurrent treatment with another doctor as well as about the taking of medication (prescribed by the physician or independently acquired).

Reuse of samples taken

The samples obtained could be of great value at a later stage and beyond the scope of the research questioned in this project. Re-use of your samples for further research questions requires your consent, which is why we ask you to communicate your wishes in the following.

A prerequisite for the use of biomaterials and data for a specific research project is that the research project has been evaluated by an ethics committee.

I agree to the continued use of the blood and urine samples obtained for research purposes

- ☐ I agree.
- ☐ I do not agree

The reuse is carried out in

- ☐ (1) pseudonymized (from the pseudonym only the student can deduce your identity)
- ☐ (2) anonymized (the samples are marked so that an assignment to your person is no longer possible)
- ☐ for all study possibilities.
- ☐ for all studies that are in the context of the questionnaire for which the material was originally donated.

_____ _____

(Place, date) (Signiture of participant)

Ich bestätige hiermit, dass ich den Probanden mündlich und schriftlich über die Ziele, den Ablauf der Studie, über die zu erwartenden Wirkungen, über mögliche Vor- und Nachteile sowie über eventuelle Risiken informiert habe. Der Proband hat seine Teilnahme durch seine persönliche Unterschrift mit Datum bestätigt.

_____ _____

(Place, date) (Signiture of study doctor: Dr. Gunther Adler)

_____ _____

(Place, date) (Signiture of study leader: Prof. Dr. Jan Frank)

Data protection consent to the study

"The effect of simultaneous oral administration of zinc and iron supplements on the bioavailability of β-carotene"

In the scientific study, personal data and medical findings are collected about you. The dissemination, storage and evaluation of these study-related data requires the following voluntary consent before participation in the study:

I agree that data collected in the course of this study are recorded on questionnaires and electronic data media and passed on to the client of the study for scientific evaluation in a pseudonymized form (ie, no names, initials or the exact date of birth in the encryption code). In the case of cancellation of the consent (cancellation of the study participation), you may request the deletion of the data collected up to that point. In the case of the publication of the study results, the confidentiality of my personal data is also ensured, if at all, the data are used in encrypted form.

_____

(Place, date)

_____

(Signiture of participant)