

CAROPROT

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Influence of proteins on carotenoid digestion and aspects of bioavailability

Statistical Analysis Plan (SAP)

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SAP contributors:

Torsten Bohn

Principal Investigator, LIH

Manon Gantenbein

Principal Investigator, LIH

Michel Vaillant

Head of CCMS, LIH

Anna Schritz

Biostatistician, LIH

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1. Introduction

1.1 Background and rationale

Health benefits and interest of carotenoids

Carotenoids are a group of C-30 to C-50 terpenoids found predominantly in many plants and several types of fungi, which are able to produce them. A limited number and amount of carotenoids can also be found in animals, such as in salmon, which accumulate these pigments in their tissues, due to their feeding habits. However, as humans cannot produce carotenoids, diet is the only source of these lipophilic compounds. Around 40-50 carotenoids have been reported to be important for the human diet [1].

Several of these carotenoids, including alpha-and beta-carotene, as well as beta-cryptoxanthin can be metabolized by the human body into vitamin A, and are thus crucial sources of this essential nutrient for vegetarians/vegans, and people not consuming much meat (which is the best dietary source of preformed vitamin A), such as in developing countries. The most predominant natural dietary sources of carotenoids include colored fruits and vegetables, such as bell peppers, tomatoes, or carrots, and green leafy vegetables such as spinach or kale [2, 3]. The consumption of carotenoids within these fruits and vegetables, as well as their blood plasma levels, have been related to the reduction of several chronic diseases, including cardiometabolic complications [4, 5] and even reduction of total mortality [6]. In addition, some carotenoids, especially zeaxanthin and lutein, are associated with reduced severity of age-related macular disease, the major cause of blindness in the elderly [7, 8]. The mechanisms contributing to these beneficial health effects are not always completely understood, but have been suggested to rely in part on the potential to influence cellular signaling cascades, reducing inflammation [9, 10], strengthening the body's own antioxidant system [11], impacting intercellular gap-junction communication [12] and regulating apoptosis [4, 13].

Despite that these noted effects are generally ascribed to the intake of carotenoids within the normal diet, it also has to be stated that these health effects could not be confirmed in studies serving isolated carotenoids at higher doses, such as in form of supplements [14, 15]. These surprising results have been explained by the hypothesis that isolated antioxidants, when supplemented at high doses, behave differently than when served within whole fruits or vegetables, where they are occurring together with additional, perhaps synergistically acting anti-oxidants, such as vitamin C and E, and may cause adverse effects especially less healthy subjects including smokers. 3

Despite the still ongoing controversial discussion on their bioactivity, carotenoids are still advertised as health promoting compounds, and found in many dietary supplements, either alone or combined with other nutrients in multivitamin/ multimineral supplements.

The bioavailability of carotenoids

Before reaching their place of action and storage, carotenoids have to go through processes and pathways that are involved in the release from food matrix, digestion, absorption, plasma transport and tissue uptake. The amount of carotenoids released from the food matrix, absorbed and available for physiological functions defines the bioavailability of an individual carotenoid [16]. The percentage of ingested carotenoids that are potentially available for absorption, i.e. bioaccessibility, depends on the release from the food matrix during digestion and its solubilization, i.e. emulsification [16].

While data with respect to carotenoid intake is frequently available, much less is known on dietary factors impacting their bioavailability, including a) changes during gastro-intestinal digestion and b) uptake by the gastro-intestinal epithelium, among others. In general, carotenoid absorption is low (usually ca. 5-20%), owing to the fact that these liposoluble micronutrients and phytochemicals need to be emulsified prior to their small intestinal uptake.

Among the factors that are known to improve carotenoid absorption are dietary lipids [17], usually attributed to enhanced micellarization of carotenoids, and the negative impact of dietary fibers, possibly inhibiting the release of carotenoids from the matrix or due to increased viscosity [1, 18]. Recently, and because of another research project in Luxembourg the potential negative effects of divalent minerals on carotenoid bioaccessibility/bioavailability have been highlighted [19-21].

Role of proteins and relation to carotenoid bioavailability

A factor that so far has received very little attention is proteins. It has been speculated that proteins can aid in emulsifying apolar dietary constituents during digestion [22, 23]. In fact, proteins proved to be effective emulsifiers by having both hydrophobic and hydrophilic groups and reduce oil-water interfacial tension by adsorption to lipid droplet surface [24]. In this respect, proteins go through a structural modification (secondary and tertiary structure rearrangements) in such a way that maximum interaction between hydrophobic segments and the hydrophilic phase is ensured [23, 25]. In addition, it has been reported that the presence of surface active compounds in food matrix constitutes one of prominent factors influencing carotenoid bioavailability [23]. Therefore, the aggregation of adsorbed protein molecules can form protective viscoelastic surface layers and prevents aggregation of lipid droplets (electrostatic and steric repulsion), thus determining emulsion stability [24] required for the formation of micelles. To our knowledge, the interaction of proteins with carotenoids during digestion, absorption, and further biodistribution, targeting to study carotenoid bioavailability, has never been studied systematically. However, we have seen previously *in-vitro* that carotenoids digested with milk resulted in higher bioaccessibility compared to carotenoids digested with oil, which – so our hypothesis – was due to the influence of emulsifying proteins during digestion [26]. Furthermore, preliminary but unpublished results in our own laboratory indicate that, for example, whey proteins can enhance beta-carotene bioaccessibility by a factor of two. This hypothesis has resulted in an accepted FNR-CORE grant (CAROPROT, C16/BM/11320230).

While these *in-vitro* experiments cited above represent a good starting point for hypothesis building and screening, it can only to some extent predict *in-vivo* conditions, and to validate the experimental results obtained we envision a human proof-of-concept nutritional study. Therefore, different proteins and concentrations will be tested and compared *in-vitro* (including whey protein isolate, soy protein isolate, caseinate sodium and gelatine), then two different proteins will be selected for human trial, using up to half of the recommended dietary allowance (RDA) of 60 g/d for adults [27].

1.2 Objectives

The main objective of the study is to investigate the influence of co-consuming two different protein sources on the bioavailability of dietary carotenoids by conducting a postprandial cross-over study.

2. Study Methods

2.1 General procedure

The ITT population is all patients randomized to a diet or control arm whatever the arm they were finally allocated. The Per Protocol population is all patients randomized and allocated to an arm that followed strictly the protocol. Participants with major deviations to the protocol will be excluded from the PP population.

For all other procedures, it is referred to the “study protocol”.

2.2 Primary Endpoints

The main endpoint of the study is the difference in AUC (concentration over time in %) of circulating carotenoids in the plasma triacylglycerol-rich lipoprotein (TRL) fraction between two different protein

sources and control (no protein added). These investigated circulating carotenoids will be lycopene and beta-carotene.

More specifically, the data will be evaluated as follows:

- a) Determination of the AUC, using the trapezoidal method (Excel)
- b) Verifying whether data is normally distributed. If not, log-transformation will be attempted.
- c) Data will be baseline subtracted – conc. at time 0 will be subtracted from all later values. Alternatively, in the frame of a sensitivity analysis, original data will be used (with baseline concentrations as covariate).
- d) Negative concentrations at time points, if occurring, will be treated as “negative area”, i.e. this area will be subtracted from the positive area.
- e) The baseline conc. will be included as a co-variate in the model.

2.3 Secondary Endpoints

Secondary endpoints will include the following:

- a) In addition to carotenoids, triglycerides will be investigated, also as the area-under time curve, though based on plasma samples (not plasma TRL fraction).
- b) Total carotenoids as the sum of individual concentrations of all carotenoids measured in the plasma triacylglycerol-rich lipoprotein (TRL) fraction will also be analysed through the calculation of the AUC
- c) timely appearance of carotenoids in the plasma-TRL fraction, i.e. their kinetics, including e.g. C_{max} , t_{max} .
- d) Another secondary endpoint will be carried out through the collection of complete fecal samples in a small number of subjects – two subjects will be selected based on voluntary availability. The purpose of this pilot sub-trial is to detect potential (colonic) carotenoid degradation products such as apo-carotenoids as well as non-degraded native carotenoids, as more polar metabolites of carotenoids have been associated with health benefits. However, no data on colonic metabolites is available so far [28]. This pilot sub-trial could be used as a later base for a follow-up study, should interesting metabolites be measured at biologically relevant concentration. In addition to the measurements of metabolites, six participants will be selected –also based on voluntary availability- to take an aliquot from their stool in order to characterize the microbiota present within the collected samples by 16S rDNA gene sequencing or similar tools.

2.4 Trial design

This is a post-prandial double blinded, randomized and controlled cross-over study with 3 periods and six sequences.

A 6x3 Williams Crossover design is applied for the six sequences. Equal allocation of six patients per sequence is planned. Patients meeting the inclusion criteria will receive three different interventions (2 different meals with added protein sources and one control meal where no protein is added) at three different time points following a one-week washout period.

| | | |
|---|---|---|
| A | B | C |
| B | C | A |
| C | A | B |

| | | |
|---|---|---|
| C | B | A |
| A | C | B |
| B | A | C |

Figure 1: 6x3 Williams Crossover Design

2.5 Randomization

A block randomization for six different blocks with a block size of four participants will be performed in R using R-package randomizeBE (version 0.3-4) and function RL4. Randomization will be conducted according to SOP OP-DMS-001 "Randomisation of Studies and un-blinding of data".

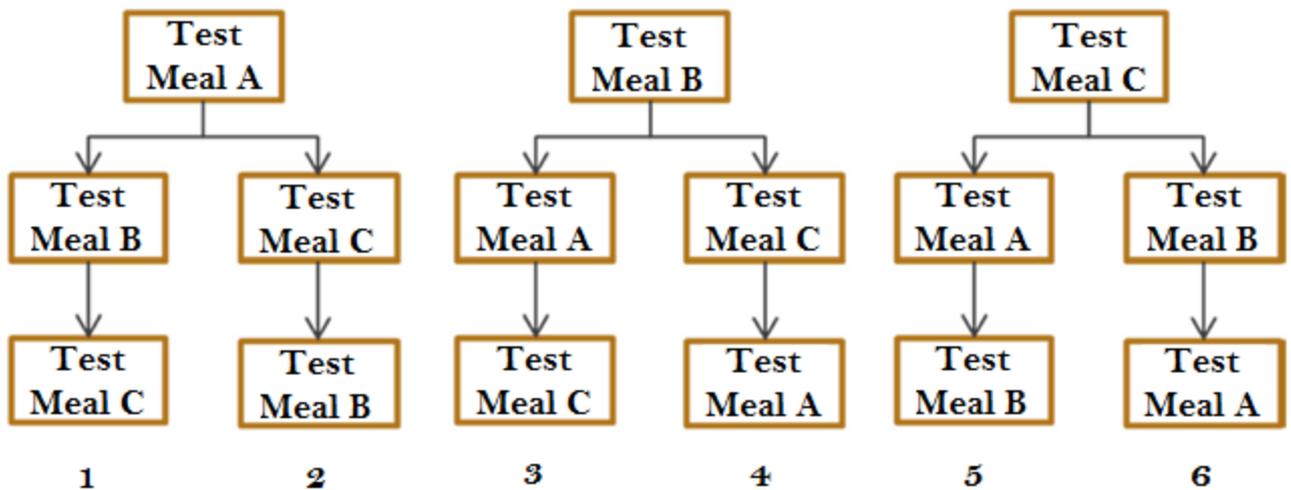


Figure 2 Schema of test meal sequences for each treatment pattern during the trial phase.

2.6 Sample Size

It is assumed that two different outcomes will be tested as dependent variables and all pairwise comparisons will be performed. First lycopene and then beta-carotene will be tested. This will lead to three statistical tests per carotenoid readout: with the original alpha risk of 0.05 the corrected alpha risk would then be $0.05/6=0.083$ i.e. 0.83%. The Bonferroni adjustment will be used to keep the family-wise error at the specified error level and thus with the total number of pairwise comparisons equal to 6 each pairwise test is at the two-sided 0.83% significance level.

Area under the time – carotenoid concentration is assumed as main outcome for the sample size calculation.

When there are 2 outcomes (lycopene and beta-carotene), 3 treatments and a sample size of 4 in each of the 6 sequences, a 6x3 Williams Crossover inequality test of paired differences will have 96% power to detect a minimum difference of 4% of the area under time curve (AUC) (difference between 20% and 24% of AUC) or greater, assuming that the standard deviation of the paired differences is 4% at the 0.83% significance level. This leads to a total sample size of N=24 participants.

In case of one drop-out per sequence (n=3 per sequence), the power will reduce to 83%, which will still provide sufficient statistical strength. Sample size calculation was performed in nQuery 8 (version 8.3.1.0).

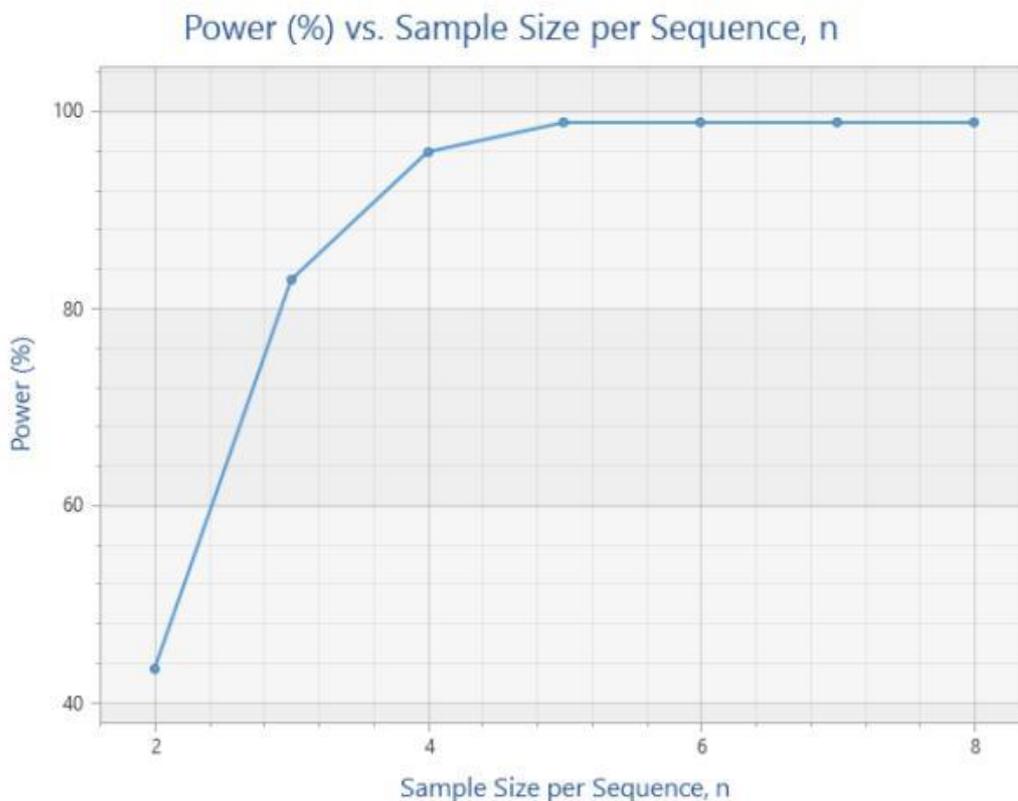


Figure 3 Power (%) vs. Sample Size per Sequence; minimum difference = 4%, SD = 4%, significance level = 0.8%



Figure 4 Power (%) vs. Minimum Expected Difference; n per sequence = 4, $SD = 4\%$, significance level = 0.8%

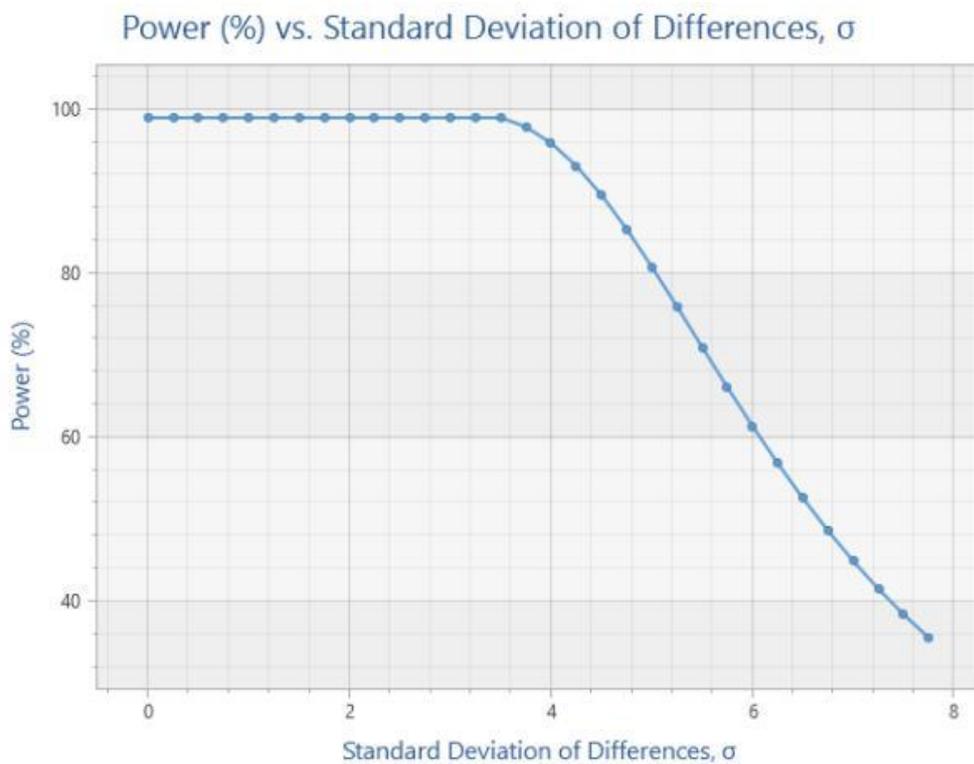


Figure 5 Power (%) vs. Standard deviation; $n=4$ per sequence, mean difference = 4%, significance level = 0.8%

2.7 Framework

A superiority hypothesis testing framework is assumed. For the main outcomes, the following comparisons are planned:

a) Difference in AUC (concentration over time) of circulating carotenoid (lycopene) in the plasma TRL fraction:

1. Protein containing meal 1 vs. control meal
2. Protein containing meal 2 vs. control meal
3. Protein containing meal 1 vs. protein containing meal 2

b) Difference in AUC (concentration over time) of circulating carotenoid (beta-carotene) in the plasma TRL fraction:

4. Protein containing meal 1 vs. control meal
5. Protein containing meal 2 vs. control meal
6. Protein containing meal 1 vs. protein containing meal 2

As a secondary outcome:

c) Difference in AUC (concentration over time) of triglycerides in the plasma:

1. Protein containing meal 1 vs. control meal
2. Protein containing meal 2 vs. control meal
3. Protein containing meal 1 vs. protein containing meal 2
- 4.

d) Difference in AUC (concentration over time) of total carotenoids in the plasma:

5. Protein containing meal 1 vs. control meal
6. Protein containing meal 2 vs. control meal
7. Protein containing meal 1 vs. protein containing meal 2

2.8 Timing of final analysis

All outcomes will be analysed collectively after the end of the study (12-18 months after study start).



Figure 6 Time plan of the whole study and duration of the individual study phases. Trial refers to the time of whole-day clinical visits of the subjects to the CIEC. Extended period of recruitment and enrolment are optional, depending on the successful recruitment of the targeted 24 male subjects.

2.9 Timing of outcome assessments

Blood samples for outcome assessment will be collected at the following moments of the study:

- a) at time point 0h on visit days and
- b) post prandial timed blood draws (time points: 2h, 3h, 4h, 5h, 6h, 8h, 10h after meal intake).

3. Statistical Principles

3.1 P- values and multiplicity adjustment

As two statistical tests will be performed per main outcome (beta-carotene and lycopene), the total number of comparisons will be 6 (3 x 2) and therefore multiplicity correction will be applied. A Bonferroni corrected p-value will be used at that level: $\frac{\alpha}{2} = \frac{0.05}{2} = 0.025$. For the three post-hoc tests within each outcome (test meals vs. control), Tukey's post hoc test will be applied.

3.2 Adherence and protocol deviations

The adherence to the intervention will be assessed based on the combined entry in the participant's study log-book, which each participant will fill out. Deviations will be discussed between the PI, CIEC and statistician and decisions for removal of subjects and collected data from the study be discussed on a case-by-case scenario. Deviations will be :

- a) Not fasting at least 10 h overnight
- b) Eating something in the morning before any of the test-meals served at the LIH.
- c) Not consuming the entire test meal within a maximum of 30 minutes.
- d) Not conducting any washout before the study.
- e) Dropping out after the first clinical day

When subjects have completed successfully at least 2 clinical visits (any 2 out of 3), their data will be analysed, if the permission from the subject is granted.

Minor deviations from the protocol that will therefore be tolerated (not dropping out):

- Drinking water during the overnight fasting
- Eating carotenoid containing foods during the washout-period, as long as the individual food items in question are listed in the volunteer's booklet
- Up to 2 missing time-points (any 2 out of 8) during the clinical days.

3.3 Analysis population

The population is healthy adult males, living in Luxembourg, and the intervention will follow the intention to treat standard. The ITT population is all patients randomized to a diet or control arm whatever the arm they were finally allocated. The Per Protocol population is all patients randomized and allocated to an arm that followed strictly the protocol. Participants with major deviations to the protocol will be excluded from the PP population.

4. Trial Population

4.1 Eligibility

Twenty-four male individuals from or living in the region of Luxembourg will be recruited for this study. The reason for recruiting male subjects only is that carotenoid plasma concentrations are likely influenced by the monthly cycle of women [29], and we preferred not to undergo any risk in this regard – this position was also accepted by the FNR (Grant CAROPROT, C16/BM/11320230).

Inclusion criteria:

- healthy and free living;
- men;
- age between 20 and 50 years old;
- Body-Mass-Index (BMI) <30 kg/m²
- non-smokers (abstinent for more than 2 years);

Exclusion criteria:

- suffering from any metabolic disease that may cause digestive disturbances (such as Crohn's disease or colitis);
- malabsorption disorders;
- BMI over 30 kg/m²;
- hyperlipidaemia (triglycerides and total cholesterol over 200 mg/dl)
- any individuals following a special diet that is not compatible with wash-out periods or test meals (vegetarian, gluten-free or diabetic);
- regular consumption of more than 5 portions (80-100 g) of fruits and vegetables per day;
- being on medical treatment or consuming any medication for chronic conditions or recent illness (e.g. antibiotics);
- consuming regularly dietary supplements;
- abnormally high or low values of plasma circulating carotenoids;
- tobacco smoking;
- frequent alcohol consumption (over 2 glasses per day);
- food allergies or intolerances that are not compatible with test meals (e.g. gluten or milk intolerance);
- daily practice of intense physical activity of 120 min or more.

No special population group such as prisoners, children, the mentally disabled or groups whose ability to give voluntary informed consent may be in question, will be recruited for this study.

4.2 Recruitment

Recruitment period

Three to twelve months will be dedicated to the recruitment of the candidates for the study. However, this period might have to be extended if the initial foreseen period is not sufficient to recruit the total number of participants or in case of a high percentage of drop-outs.

During the recruitment period, male subjects interested in participating in the study will have the opportunity to register at the CIEC by phone, e-mail or regular mail. The staff of the CIEC will contact the registered candidates by phone to briefly explain the project, schedule an appointment for an information session, and send out a copy of the "Participants' information sheet" so that interested candidates have the opportunity to carefully read the project's description and experimental procedure, prior to signing the "Informed Consent Form".

Enrolment period

The enrolment period will develop over a foreseen period of up to twelve months, with the possibility of extending this period in case of necessity. The enrolment period will involve: a) a session where

selected participants will be fully informed about the study, have the opportunity to pose any questions they might have, sign the “Informed Consent form” and fill out the “Health&Lifestyle questionnaires”; b) the selection of eligible candidates based on the results from the questionnaires; and c) a screening visit to collect a blood sample (20 ml) and urine sample (ca. 10-20 ml).

4.3 Withdrawal/ follow-up

Participants are free to withdraw from the study at any moment if they desire to do so. However, we will try our best in terms of follow-up to keep subjects in the study. Any participant showing new medical problems that are not easily explained (e.g. colds) or that requires the use of medication (e.g. antibiotics) will be withdrawn from the study.

Subjects that have at least completed 2 clinical days will be kept for statistical analysis, and given that subjects agree to keeping their data. Otherwise, the cases will be removed from analysis.

4.4 Baseline patient characteristics

Anthropometric parameters

During the enrolment phase, the candidate will be measured for the following:

- height
- weight
- waist and hip circumference
- waist-to-hip ratio
- percentage of body fat as calculated by bioelectrical impedance analysis (BIA)

Urine sampling

During the enrolment phase, candidates will be asked for a urine sample for screening of any metabolic disorder that might be an exclusion criterion (e.g. diabetes) and that the candidate is not aware of or was not mentioned in the Health&Lifestyle questionnaire.

Urine will be tested for standard urine analysis parameters such as

- Glucose
- Ketones
- ascorbic acid
- pH (GAK Combi Screen Plus 9, Analytikon, Hennigsdorf, Germany)

Blood sampling

Blood samples will be collected during the enrolment phase; prior to the beginning of the initial washout period and at time point 0h on visit days.

Continuous baseline variable will be described using the total number of samples, mean, SD (standard deviation) and median for the total sample. Categorical and binary variables will be described by number of cases in each category and percentage.

5. Analysis

5.1 Outcome definition

Primary outcomes:

Beta-carotene and lycopene

Beta-carotene and lycopene will be measured in the plasma triacylglycerol rich lipoprotein (TRL) fraction in μM at baseline (0h) and 2h, 3h, 4h, 5h, 6h, 8h, 10h after test meal intake. Using non-compartmental analysis, repeated measures over time of carotenoids will be summarised as postprandial AUC (trapezoidal approach) using the PK functions plug-in for Microsoft Excel and will be validated in a statistical software such as R or SAS system. Of note, it is planned to baseline-subtract all further concentrations (hours 2-10), and to also subtract potentially occurring negative values from the AUC.

Baseline concentrations will be included as co-variates into the linear mixed model.

Secondary outcomes:

Triglycerides

Triglycerides will be measured in the plasma in mg/dL at baseline (0h) and 2h, 3h, 4h, 5h, 6h, 8h, 10h after test meal intake. Using non-compartmental analysis, repeated measures over time of triglycerides will be summarised as postprandial AUC (trapezoidal approach) using the PK functions plug-in for Microsoft Excel and will be validated in a statistical software such as R or SAS system.

Minor abundant carotenoids:

Isomers of beta-carotene and lycopene will be treated as the all-trans native form, i.e. all chromatographic peak area will be combined if isomers are identified in the plasma-TRL fraction.

If alpha-carotene or other carotenoids such as lutein and beta-cryptoxanthin can be measured (if chromatographic sensitivity is sufficient), AUC in the TRL fraction will also be determined, as described for beta-carotene and lycopene.

They will be accounted for in the analyses as secondary outcomes with similar analysis methodology

Total carotenoids will be produced by summing up all carotenoids measured and analysed as the primary endpoint

Carotenoids after colonic digestion:

Native carotenoids after colonic digestion, as well as excreted degradation products such as apo-carotenoids will be investigated, as far as the analytical capabilities allow. As no previous data exists in this respect, this part will be of highly explorative character.

5.2 Analysis methods

The primary endpoint, AUC of either lycopene or beta carotene will be analysed using a linear mixed model using randomization sequence and treatment (test meal) as fixed terms and patient within sequence as a random term taking into account the cross-over design as described in chapter 5 of "Cross-Over Trials", Senn (2002). Baseline will be included as a co-variate.

Assumptions of normality and equality of variance will be verified by normality plots and boxplots, respectively. If data is non- normally distributed, log transformation will be attempted.

Non-parametrical analysis (Friedman tests followed by Wilcoxon- Mann- Whitney test) may be chosen in case data is not normally distributed, if log-transformation is not successful, though based on earlier studies data normally follows normal distribution.

The secondary endpoints - triglycerides, and if feasible additional carotenoids (see 5.1 above) will be analysed by the same methodology.

A linear mixed model will be first performed for each main outcome, with either baseline corrected AUC of lycopene or beta-carotene as the observed, dependent variable, and randomization sequence, test-meal as the independent variable (fixed factor) and patient within sequence as random factor. Pairwise comparisons of the intervention will be performed by means of Tukey's post-hoc test. Additionally, sensitivity analysis with the original outcomes (lycopene or beta-carotene) as dependent, randomization sequence, test meal and baseline concentration of the outcome as fixed factors and patient within sequence as random factor. 95% confidence intervals will be reported for the main outcomes.

5.3 Missing data

Individual time-points, if missing, during the clinical days will be estimated by using multiple imputations (Multivariate Imputation by Chained Equations).

For complete missing data-sets (1 or 2 clinical days missing), see withdrawal/follow-up.

5.4 Harms

Severe adverse effects will be noted, and inclusion of data in the final dataset will be discussed between the PI, the CIEC team and the statistician based on a case-by-case scenario. The identity of the subject will be kept confidential and not shared by the CIEC.

5.5 Statistical software

- PK functions plug-in for Microsoft Excel to calculate AUC with the trapezoidal approach.
- Data analysis will be performed with SPSS 19.0 for Windows (SPSS Inc., Chicago IL).
- R or SAS for validation

5.6 References

For statistical evaluation, please see previous publication [21]

The statistical master-file will be kept under the "P"-drive of the PI (Dr. Torsten Bohn).

SOP OP-DMS-001 "Randomisation of Studies and un-blinding of data"

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