CAROPROT – Influence of proteins on carotenoid digestion and aspects of bioavailability

Study protocol

Version date: 07.08.2019

1. TITLE

CAROPROT- "Influence of proteins on carotenoid digestion and aspects of bioavailability".

2. PROMOTERS

Epidemiology and Public Health Research Unit (EPHRU) and Clinical and Epidemiological Investigation Center (CIEC) of the Luxembourg Institute of Health (LIH).

3. PRINCIPAL INVESTIGATORS

Dr. Torsten Bohn (EPHRU)

Dr. Manon Gantenbein (CIEC)

4. 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.

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 as a consequence of another research project in Luxembourg, also 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 2. 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 gelatin), 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].

5. MAIN OBJECTIVES OF THE STUDY

Our objective is to conduct a postprandial study to test the influence of co-consuming two different protein sources on the bioavailability of dietary carotenoids.

6. PRIMARY ENDPOINT

The main endpoint of this study is to measure the effects of 2 different protein sources vs. the control (no added proteins) on the post prandial Area-Under-the-Curve (AUC, i.e. concentration over time curve) of circulating carotenoids in the plasma triacylglycerol-rich lipoprotein (TRL) fraction, representing newly absorbed carotenoids, similar as carried out in a recent trial in Luxembourg [21]. The values will be baseline-corrected, i.e. the first AUC value (time 0) will be subtracted from all further values, transformation of the data (e.g. log) will not be required. AUC will be determined via the trapezoidal method.

7. SECONDARY ENDPOINT

Secondary endpoints include studying the timely appearance of carotenoids in the plasma-TRL fraction (i.e. their kinetics), as well as studying the response of the triglycerides, which will be studied in the plasma. This will give us more insights about potential gastro-intestinal differences regarding passage time, which may influence absorption and bioavailability.

Another secondary endpoint will be the collection of complete fecal samples in a small number of subjects – 6 subjects will be selected at random. The purpose of this pilot-trial is to detect potential (colonic) carotenoid degradation products, as more polar metabolites of carotenoids have been associated with health benefits. However, no data on colonic metabolites is available [28]. This pilot-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, an aliquot will be taken from each collection in order to characterize the microbiota present within the collected samples by 16S rDNA gene sequencing or similar tools.

8. DURATION OF THE STUDY

Study duration for the participants

Should the participants complete the trial, the predicted individual duration of the study will be 4 weeks (see participants' schedule in Figure 3). We foresee that 12 to 18 months will be necessary for all participants to complete the entire study.

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).

Total duration of the study

Total duration of the study, including enrolment and recruitment as well as the trial itself, will be of 12 to 18 months. Please refer to Figure 1 for more details on the duration of each study phase.

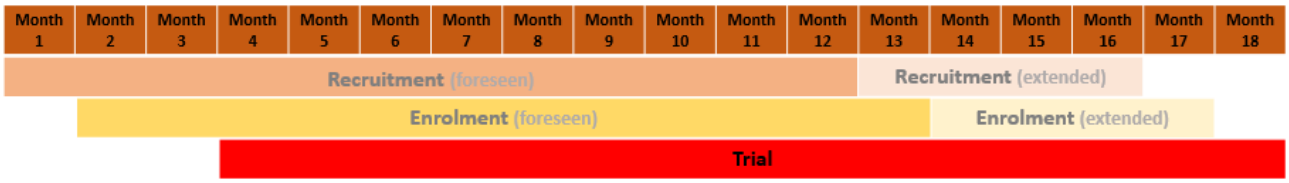

Figure 1 – Time plan of the whole study and duration of the individual study phases. Trial refers to the time of whole-day clinical visits of the subjects to the CIEC. Extended period of recruitment and enrolment are optional, depending on the successful recruitment of the targeted 24 male subjects.

9. PARTICIPANTS

Number of Participants

Twenty four male individuals from or living in the region of Luxembourg will be recruited for this study. The number of participants is based on a randomized block design, constituting of 6 blocks of 4 subjects, each will be served different sequences of test meals (A, B, C/A, C, B/B, C, A/B, A, C/C, A, B/C, B, A) in order to cancel out any potential effect of sequence of meals (See scheme of test meal sequences in Figure 2).

Our aim would thus be to recruit 24 subjects, and try our best in terms of follow-up and interaction to keep them in the study. However, should several subjects opt to leave the study, we would still, even with only ca. 15 subjects, reach

sufficient statistical strength to see small differences in absorption in this cross-over study. For example, a difference in fractional absorption between 10% and 8% could be resolved already in a pairwise design with a SD of absorption difference of ca. 2.5% for an alpha of 0.05 and a power of 80% (http://biomath.info/power/prt.htm). As each subject will act as his/her own control, this could be seen as a realistic estimate. 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/m^2
- 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/m^2;
- 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.

10. STUDY PROCEDURES

Recruitment

Candidates will be informed of the study by resorting to flyers, newspapers, e-mails, "word of mouth" communication and advertisement at the LIH website for general public.

Flyers will be placed on a variety of locations throughout the different campuses (Limperstberg, Belval and Kirchberg) of the University of Luxembourg, Centre Hospitalier du Luxembourg, LIH, student's residences and other different locations.

The study will also be advertised via e-mails that will be sent internally to colleagues at the LIH, and externally to the students and staff network of the University of Luxembourg.

"Word of mouth" seems to be, still, one of the most effective ways to advertise events. Hence, communication about the human trial will be spread among students, friends, faculty staff, work colleagues and other social contacts.

Furthermore, advertisements might be placed in some local newspapers such as L'Essentiel, Tageblat, Contacto and Wort.lu.

Candidates may contact the CIEC or a responsible researcher to schedule a day to fill out Health&Lifestyle questionnaires and to collect a blood and urine sample.

Recruitment procedures will not include, at first, women in view of the influence of the menstrual cycle on women's metabolism, which may ultimately introduce a source of error to the results.

However, should we fail to attain the desired number of participants (24) in the first wave of recruitment; we will promote a second wave of study advertisement recruiting women only. In this case, it will be aimed to have a balanced study with approx. 12 men and 12 women, if possible.

Enrolment

To evaluate the eligibility to participate in the study, each candidate will fill out a Health&Lifestyle questionnaire, and each candidate who is willing to enlist will be asked for a blood and urine sample. These samples will be used to screen for any metabolic disorder, risk of anaemia, abnormally high or low levels of plasma carotenoids, or any other factor that may make the candidate not eligible for the study.

Selected candidates will undergo an informed consent process during which they will be educated individually about the goals, procedures and possible risks of the study. The participant will receive a copy of both the "Informed Consent" and the "Participants Information's sheet". Also a list of foods to be avoided during the washout-out period, together with a second list of alternative foods will be given to each participant (see attachment). The participant may sign the consent form at the initial evaluation, or he/she may take a copy home for further consideration and sign it at a later visit to enroll in the study.

Experimental Design

This is a human proof-of concept, post-prandial randomized cross over study. Twenty four, free-living human participants (age 20-50 years, male) will be recruited. A placebo-control is not possible in sight of the amount protein that will be consumed, and the lack of a surely negative control that could be given instead of proteins and that would not be distinguishable from proteins in terms of color, taste and texture.

All participants will be instructed to avoid foods rich in carotenoids before clinical visits. On clinical visit days, participants will be asked to report at the CIEC's facilities, starting from 7:30 am, and a baseline blood sample (20 ml) will be drawn at 0h time point. A trained nurse will insert a cannula in the forearm of the participant that will be left in place during the whole staying for the commodity of the participant.

Immediately after the baseline blood draw, a test meal composed of a mixture of carrot and tomato juice (350 mL in total), to which 5 mL of peanut oil will be added, 40 g of toasted bread (white wheat, with 10 g margarine plus 20 g cream-cheese) and a glass of water (approx. 300 mL) which may or may not contain 30 g of proteins (either a plant-

based protein or a dairy-based proteins) will be served. The entire test meal must be eaten within 30 min, under supervision.

Post-prandial blood samples (20 ml each) will be collected at timed intervals (before, 2h, 3h, 4h, 5h. 6h, 8h and 10h after test meal intake). Participants will receive a standardized lunch 4 hours after test meal intake (c.a. 12:00 pm), consisting of a toasted sandwich (white wheat bread, ca 60 g), with ca. 60 g turkey with some margarine to spread on the bread (ca 10 g), a Greek yogurt (140 g) and a small apple. A courtesy meal 10 hours after test meal intake for dinner and at the end of the visit. No other foods or beverages except water (*ad libitum*) will be allowed during the day (including during breakfast and lunch if desired).

Stool collection: For a limited number of subjects (n=6), the samples will be collected following intake of all 3 test meals. For this purpose, Containers for collection and plastic clip-bags and bags will be handed to participants. Boxes with cooling elements for stool collection will likewise be given. Also, six fecal color markers will be provided to the participants. For each clinical visit, two fecal marker are needed. Collections will start from the excretion of the first fecal color marker (brillant blue) which will be taken at the beginning of the fasting period, i.e. the day before each clinical day. The collection will continue to the excretion of the second fecal marker which should be taken on the morning of the day following each clinical visit (before breakfast). Samples will be returned after the clinical test day as soon as the last fecal marker has been excreted (normally resulting in 2-3 day complete fecal samples), and could be collected once in between by staff from the LIH if needed (See representative scheme of the participants' schedule in figure 3).

Randomization

In short, 3 test meals will be given to the participants in 6 different orders, making 6 treatment patterns (Figure 2). Assuming that all 24 participants are successfully recruited, we will have 4 randomly allocated participants per pattern.

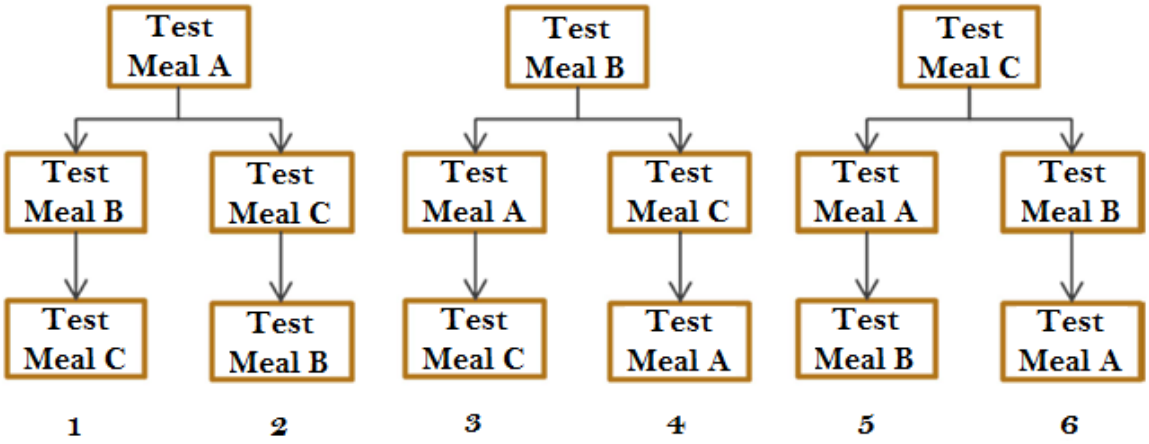

Figure 2 – Schema of test meal sequences for each treatment pattern during the Trial phase.
(Test meal A: No added protein, Test meal B: 1st protein added, Test meal C: 2nd protein added)

Participants' Schedule

During the recruitment phase, the subjects who show interest in participating in this study will undergo an information session with the study researchers. This session is meant to inform the participant about the trial, answer all of his questions, sign the Informed Consent Form and fill out our Health&Lifestyle questionnaire, which is used to determine the eligibility for this study.

Participants who agree to sign the Informed Consent Form and are considered eligible for participation will be scheduled for a first and brief screening visit (enrolment visit) to collect a spot urine and blood sample (20 ml) that are meant to check the subjects anemic status and analyze the sugar levels as well as plasma lipids such as triglycerides, in order to screen for any abnormal health condition (e.g. onset of diabetes) that they might not be aware of at the date of the information session and that might not make them eligible for this study.

If at this stage they are still considered eligible for the study, participants will commence the 4 week trial phase which includes 3 washout periods, 1 short screening visit and 3 full day (i.e. 10.5 hours) clinical visits. The screening visit, called preliminary visit, will take place at the beginning of the trial phase. A blood sample will be collected to determine the baseline levels of triglycerides and plasma carotenoids at the beginning of the trial, prior to the first washout phase.

The first washout week starts on day 1 after the preliminary visit and will have a duration of 14 days during which the participants will be asked to stay on a low carotenoid diet (i.e. to avoid the intake of colored fruits and vegetables), to reduce the basal levels of blood circulating carotenoids. This first appointment will be followed by a 1 week washout period, during which he/she will continue on a low carotenoid diet (3rd week of washout diet). At the end of the 3rd week of washout, the second appointment at the CIEC will take place. The procedure for this day is the same as mentioned above. The second appointment is followed by the 4th and last washout week and at the end the participant will have the 3rd and last appointment at CIEC, which will be identical to the other visits. For all washout periods the participants will be asked to fill a provided food journal, on a daily basis, where they will write down what they have eaten during the day. This will be used to check compliance with the washout period and better interpret personal data.

Please find in Figure 3 a scheme of the participant's schedule during the trial phase.

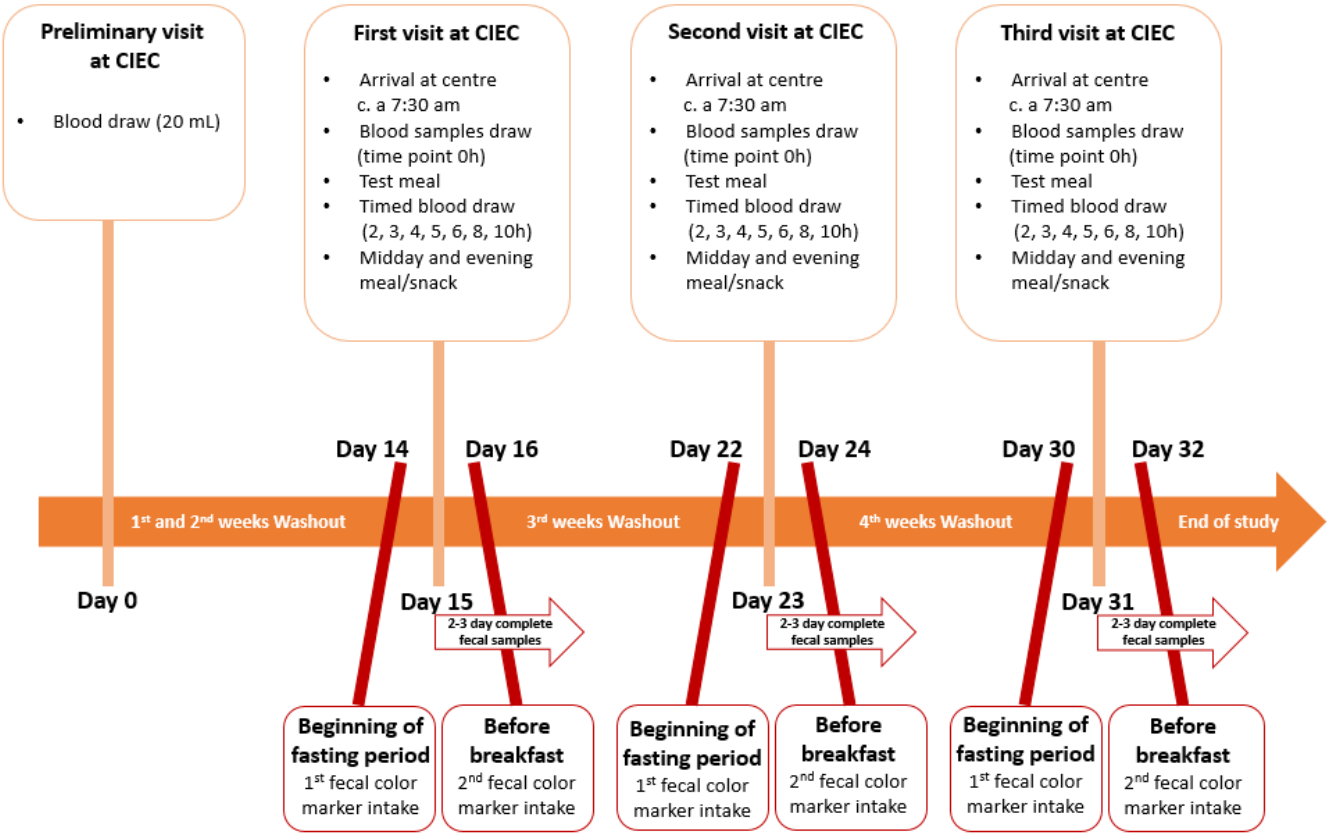

Figure 3 – Representative scheme of the participants' schedule during the trial phase.

Data management:

The data collected in this study (lifestyle questionnaires, urine and blood samples, carotenoid plasma and plasma-TRL fraction analysis -a special fraction isolated from blood plasma-, biometric measures) will be used to investigate the influence of dietary proteins on the bioavailability, that means uptake and usage of carotenoids by the human body. The results of the investigation will possibility be published in international scientific journals without detriment to the subject's privacy.

The data collected will be treated in a strictly confidential manner and will be kept up to five years after completion of the study. The processing of the data will be done by means of a code (pseudonimisation). This code does not identify participants and will only be used for the scientific treatment of the results.

According to the provisions of the amended Grand-Ducal Law of 2nd August 2002 on the protection of individuals with regard to the processing of personal data, a notification will also be submitted to the 'Commission Nationale pour la Protection des Données' (CNPD).

Insurance:

The LIH will subscribe an insurance before the launch of the study. It will be insured to indemnify the participant who suffers from any claim for damage injury that happened on the clinical visit or on the way to it.

Research Methodology

Dry Run

Prior to the beginning of the trial phase we will perform a "dry run", i.e. a test run, to test the study's methodology, evaluate possible pitfalls and critical control points and optimize the protocol if needed.

This will include: a) filling out of the Health&Lifestyle to estimate the total amount of time needed to complete the questionnaire and determine the suitability of the questions; b) following the washout diet and c) one full day clinic visit to evaluate the logistics during clinical visit days and test the protocol for the extraction of the plasma TRL fraction and measurement of plasma carotenoids. The "dry run" will include 2 volunteers, and will be recruited from staff of the LIH. Volunteers for the dry run will not receive monetary compensation.

Test Meals

There will be 3 test meals, each one composed of a standard portion, comprising a carotenoid-rich ingredient (mix of predominantly carrot and tomato juice 1:1, v/v, 350 mL total, plus 5 mL peanut oil), 1 toasted slice of bread (40 g white wheat, with ca. 10 g margarine plus cream cheese (ca. 20 g, 17.5% fat), and a glass of water (300 mL) containing or not 30 g of dissolved protein supplement (either a plant-based protein or a dairy-based proteins).

Table I – Composition of Test Meals

Test Meal	Standard portion (Ingredients and amounts)	Protein supplement (30 g) dissolved in test meal
1	350 mL of mix carrot and tomato juice plus 5 mL peanut oil.	None
2	40 g of toasted bread.	Type 1
3	20 g of cream-cheese plus 10 mg margarine.	Type 2

Test meal portions will be prepared in advance on the morning of the clinical visit. The meals will be prepared, from marketed products. Each meal will be prepared in a standardized fashion so that every meal is equal in calories and composition, except for the amount and type of protein.

Participants will be instructed to consume the test meals within 20 min, under supervision.

Anthropometric parameters

During the enrolment phase the candidate will be measured for the following: a) height; b) weight; c) waist and hip circumference; d) waist-to-hip ratio and e) 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, and pH (GAK Combi Screen Plus 9, Analytikon, Hennigsdorf, Germany).

Women, if enrolled for this study, under the conditions mentioned in the Study Procedures, will also undergo a pregnancy test. (Clearblue, Swiss Precision Diagnostics GmbH, Petit Lancy, Switzerland).

Blood sampling

Blood samples will be collected at the following moments of the study: a) during the enrolment phase; b) prior to the beginning of the initial washout period; c) at time point 0h on visit days and d) post prandial timed blood draws.

Please refer to Table II for details on parameters analyzed and at which phases of the study.

Table II – Parameters analyzed from blood samples collected throughout different stages of the human trial. HDL – high density lipoproteins; LDL – low density lipoproteins; TG – triglycerides; TRL – triglyceride-rich lipoprotein fraction. Post prandial blood samples during clinic visits collected at the following time points: 2h; 3h; 4h; 5h; 6h; 8h; 10h.

Parameter	Enrolment phase	Beginning of trial (start of first washout phase)	Time point 0h on clinical visits	Post prandial times on clinical visits
HDL, LDL and total cholesterol	✓			
TG	✓	✓	✓	✓
Plasma carotenoids	✓	✓	✓	✓ *
Plasma TRL carotenoids			✓	✓
Blood Glucose	✓			
Hematocrit	✓			
Hemoglobin	✓			

All parameters (Table II) will be analyzed by a private laboratory, such as *Laboratoires Reunis*, with the exception of plasma carotenoids and plasma TRL carotenoids, which will be analyzed at the LIH.*plasma samples will be kept as a back-up for potential additional analyses.

Isolation of blood plasma

Blood samples will be collected from a forearm vein into an EDTA-containing tube and immediately centrifuged for plasma separation at 1250 x g for 10min at 4°C, which will be then stored at 4°C for later analysis.

Isolation of triglyceride-rich lipoprotein (TRL) fraction

TRL fractions will be isolated via ultracentrifugation of previously isolated blood plasma. For this purpose, 3.0 mL of plasma will be overlaid with 2.0 mL NaCl solution (density 1,006 g/L) and ultra-centrifuged at 100 000 x g at 4°C for 30 min at 20°C. TRL fraction (0.6 ml) will be standardized to 1.5 ml with saline, flushed with nitrogen and stored at -80ºC until analysis [30].

Stool collection

Fecal samples will be collected from up to 6 subjects, for all 3 test meals. Six fecal color markers will be provided to the participants. For each clinical visit, two fecal marker are needed, the first marker should be taken the day before the clinical visit (at the beginning of the fasting period) and the second marker should be taken on the morning of the day after the clinical visit (before breakfast). Collections will start as soon as the first marker is

excreted and will continue until the excretion of the second fecal color marker, normally resulting in 2-3 days complete fecal samples. Also, an aliquot should be taken at the beginning of the collection, in order to charaterize the microbiota present in the collected samples, by 16S rDNA gene sequencing or similar techniques. The aliquot will be kept in the CryoShipper (used for the safe transportation of biological samples at cryogenic -150 ° C or colder). The CryoShipper will be provided to the participant if the aliquot collection has not occurred on the clinical visit day. The characterization of the microbiota is important, as it may be related with the ability to produce carotenoid metabolites. In addition, Containers for collection and plastic clip-bags and bags will be handed to subjects. Boxes will cooling elements for stool collection will likewise be given. Samples will be returned by the subject 2-3 d after the clinical test day as soon as the last fecal marker has been excreted at the very latest, or, preferably, also collected once in between by staff from the LIH. Representative aliquots of the stool samples will be prepared similar as discussed in Van Lieshout et al. [31].

11. STATISTICAL ANALYSIS

The baseline corrected AUC for carotenoids will be calculated by the trapezoidal approach, using the PK functions plug-in for Microsoft Excel. Negative values, if occurring, will be included as such.

Mean AUC between the test meals will be compared by a repeated measures ANOVA design (i.e. each subject will be compared against himself at 3 time points), followed by post-hoc test comparisons (Fisher protected LSD test) given a significant Fisher-F test. No log-transformation will be carried out, but AUC values will be baseline corrected. Thus, a model with the observed dependent factor baseline-corrected-AUC for each carotenoid (lycopene, beta-carotene) and the triglycerides will be run, with subject, randomization sequence, test meal as fixed factor and baseline carotenoid concentration as a covariate.

Normal distribution will be verified by Q-Q-plots and Kolmogorov-Smirnoff tests.

Non parametrical analysis (Friedman tests followed by individual t-tests) may be chosen in case data is not normally distributed, though based on earlier studies data normally follows normal distribution. Log-transformation will not be carried out.

P-values below 0.05 will be interpreted as statistically significant (2-tailed). Data analysis will be performed with SPSS 19.0 for Windows (SPSS Inc., Chicago IL).

12. VALORIZATION OF STUDY RESULTS

Benefits for the participant

1. Participants will be informed about their current health status as indicated by their blood biochemistry and anthropometric profile (please refer to Table II for the parameters analyzed in the enrolment phase).

2. All meals served in the trial will be free of charge.

3. Participants will receive for their participation in the study a total of 300€ and a total of 400€ as compensation if also stool samples will be collected. The value of compensation was based on similar previous trials such as the CNER evaluated BIOCAR study.

Benefits for the society in general

This nutritional postprandial study financed by the Luxembourgish FNR (*Fonds National de la Recherche*) is the extension of the work already carried out by our group within the BIOCAR study, studying the influence of divalent minerals on the bioavailability of carotenoids, which was the first nutritional postprandial study carried out in Luxembourg.

An additional asset of this study is the continued implication of the LIH's Clinical and Epidemiological Investigation Centre towards the conduction of further nutritional studies and will lay the foundation for future studies in this direction, which could move toward more long term dietary interventions, and perhaps including larger numbers of participants. Also, this would be the first nutritional study investigating the influence of proteins on the bioaccessibility and bioavailability of carotenoids.

A healthy diet should not only be of healthy composition, it is also important that potential health beneficial compounds are bioavailable, i.e. they should be absorbed to a sufficient extent from the diet to be used for specific functions by the human body. However, very little information is available on the bioavailability of carotenoids in the presence of proteins.

We are convinced that this study will allow us to obtain information about the absorption and bioavailability of carotenoids from a carotenoid-rich meal when consumed together with supplemented protein. The obtained information would be an important contribution for developing an improved healthy diet for the average consumer that would enhance carotenoid absorption and mitigate digestive losses as much as possible.

The influence of proteins on carotenoids during digestion, uptake, and absorption has never been systematically studied. However, in sight of the situation that only few food items contain both proteins and carotenoids, it would be interesting to see if carotenoid absorption can be increased when combining fruits/vegetables with protein rich meals. This would surely constitute a question of interest for many consumers, notably vegetarians/vegans, but also for subjects not consuming many animal-based foods (rich in vitamin A), who rely on vitamin A intake by carotenoid consumption, such as in many developing countries.

13. RISKS AND POSSIBLE INCONVENIENTS OF THE STUDY

Risk for subjects:

The risks associated with this study protocol in the population described above are minimal. No invasive procedures will be used in this study apart from peripheral blood drawings. There are minor risks associated with venipuncture such as mild discomfort, fainting or bruising. Less commonly, a small clot, swelling of the vein, infection, or bleeding may occur at the site of puncture. All venipuncture procedures and blood samplings will be performed by a trained nurse from the CIEC.

Participants will be asked in advance for any kind of food allergies or intolerances (e.g. gluten or milk) that are not compatible with the test meals and midday and evening snacks, given throughout the days of the trial. 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.

Indeed, allergies against various proteins, including those that can be selected, have been reported. For soy protein isolate, two proteins β-conglycinin and glycinin have been suggested to be the major fractions containing allergens [32, 33], affecting approximately 0.4% of children, making soy allergy about half as common as peanut allergy [34]. The latter allergy is caused by eleven allergenic proteins [35] and Ara H2 protein is the most potent allergen [36]. However, in refined peanut oil, this protein is not expected to be present [37]. For whey protein isolate, α-lactalbumin and β-lactoglobulin have been stated as the main allergens, affecting up to 1–3% of adults and 3–8% of infants [38] [39]. When αS1-casein seems to be a major allergen of sodium caseinate according to IgE and T cell recognition data [40], and affecting about 5-6%% of young children [41], α2 chain has been reported as a main allergen of gelatin bovine, and affecting 3% of children with a history of beef meat allergy [42]. We will carefully question the participants regarding any allergies that they may have encountered and exclude any subject with a history of allergic reactions toward peanut, soy, or milk protein.

Carotenoids in amounts similar as consumed in our study are consumed within daily diets and have been served, also within fruits and vegetables in previously in other human studies (Unlu et al. 2005; Bohn et al. 2011, Borel et al, 2017) with no observed adverse effects.

Drop-outs and power calculation:

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. However, should several subjects opt to leave the study, we would still, even with only ca. 12-15 subjects, reach sufficient statistical strength, i.e. power, to see a reasonable difference in absorption, if it exists.

Power calculation:

For example, a cross-over trial with four subjects in each of six sequences will have 95% power to detect a difference of 20% (e.g. fractional absorption 20 vs. 24%) at the 5% level (two-sided) if the SD of paired differences within subjects is 20%. A design with four subjects per sequence makes ample allowance for possible drop-outs and also for adjusting the type I error rate for individual contrasts to allow for multiplicity (http://www.quantitativeskills.com/sisa/calculations/samsize.htm). As each subject will act as his/her own control, this could be seen as a realistic estimate.

15. REFERENCES

1. Bohn, T., *Bioavailability of Non-Provitamin A Carotenoids.* Current Nutrition & Food Science, 2008. **4**: p. 240-258.
2. Scherz, H., et al., *Food composition and nutrition tables*. 6th, rev. and completed ed. 2000, Boca Raton, FL: CRC Press/Medpharm. xxx, 1182 p.
3. Biehler, E., et al., *Contribution of violaxanthin, neoxanthin, phytoene and phytofluene to total carotenoid intake: Assessment in Luxembourg.* Journal of Food Composition and Analysis, 2012. **25**(1): p. 56-65.
4. Tapiero, H., D.M. Townsend, and K.D. Tew, *The role of carotenoids in the prevention of human pathologies.* Biomedicine & Pharmacotherapy, 2004. **58**(2): p. 100-110.
5. Wang, L., et al., *Associations of plasma carotenoids with risk factors and biomarkers related to cardiovascular disease in middle-aged and older women.* Am J Clin Nutr, 2008. **88**(3): p. 747-54.
6. Buijsse, B., et al., *Plasma carotene and alpha-tocopherol in relation to 10-y all-cause and cause-specific mortality in European elderly: the Survey in Europe on Nutrition and the Elderly, a Concerted Action (SENECA).* Am J Clin Nutr, 2005. **82**(4): p. 879-86.
7. Broekmans, W.M., et al., *Macular pigment density in relation to serum and adipose tissue concentrations of lutein and serum concentrations of zeaxanthin.* Am J Clin Nutr, 2002. **76**(3): p. 595-603.
8. Cooper, D.A., *Carotenoids in health and disease: recent scientific evaluations, research recommendations and the consumer.* J Nutr, 2004. **134**(1): p. 221S-224S.
9. Elliott, R., *Mechanisms of genomic and non-genomic actions of carotenoids.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2005. **1740**(2): p. 147-154.
10. Kaulmann, A., et al., *Carotenoid exposure of Caco-2 intestinal epithelial cells did not affect selected inflammatory markers but altered their proteomic response.* Br J Nutr, 2012. **108**(6): p. 963-73.
11. Kaulmann, A., et al., *Carotenoids, polyphenols and micronutrient profiles of Brassica oleraceae and plum varieties and their contribution to measures of total antioxidant capacity.* Food Chemistry, 2014. **155**: p. 240-250.
12. Aust, O., et al., *Lycopene oxidation product enhances gap junctional communication.* Food Chem Toxicol, 2003. **41**(10): p. 1399-407.
13. Palozza, P., et al., *beta-Carotene exacerbates DNA oxidative damage and modifies p53-related pathways of cell proliferation and apoptosis in cultured cells exposed to tobacco smoke condensate.* Carcinogenesis, 2004. **25**(8): p. 1315-25.
14. Omenn, G.S., et al., *Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial.* J Natl Cancer Inst, 1996. **88**(21): p. 1550-9.
15. Gallicchio, L., et al., *Carotenoids and the risk of developing lung cancer: a systematic review.* Am J Clin Nutr, 2008. **88**(2): p. 372-83.
16. Etcheverry, P., M.A. Grusak, and L.E. Fleige, *Application of in vitro bioaccessibility and bioavailability methods for calcium, carotenoids, folate, iron, magnesium, polyphenols, zinc, and vitamins B(6), B(12), D, and E.* Front Physiol, 2012. **3**: p. 317.
17. Unlu, N.Z., et al., *Carotenoid absorption from salad and salsa by humans is enhanced by the addition of avocado or avocado oil.* J Nutr, 2005. **135**(3): p. 431-6.
18. Yonekura, L. and A. Nagao, *Soluble fibers inhibit carotenoid micellization in vitro and uptake by Caco-2 cells.* Biosci Biotechnol Biochem, 2009. **73**(1): p. 196-9.
19. Corte-Real, J., et al., *Effect of divalent minerals on the bioaccessibility of pure carotenoids and on physical properties of gastro-intestinal fluids.* Food Chemistry, 2016. **197, Part A**: p. 546-553.

20. Corte-Real, J., et al., *Negative effects of divalent mineral cations on the bioaccessibility of carotenoids from plant food matrices and related physical properties of gastro-intestinal fluids.* Food Funct, 2017. **8**(3): p. 1008-1019.

21. Corte-Real, J., et al., *No influence of supplemental dietary calcium intake on the bioavailability of spinach carotenoids in humans.* Br J Nutr, 2017. **117**(11): p. 1560-1569.

22. McClements, D.J. and Y. Li, *Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components.* Adv Colloid Interface Sci, 2010. **159**(2): p. 213-28.

23. Soukoulis, C. and T. Bohn, *A Comprehensive Overview on the Micro- and Nano-technological Encapsulation Advances for Enhancing the Chemical Stability and Bioavailability of Carotenoids.* Critical Reviews in Food Science and Nutrition, 2015: p. 00-00.

24. Qiu, C., et al., *Influence of protein type on oxidation and digestibility of fish oil-in-water emulsions: gliadin, caseinate, and whey protein.* Food Chem, 2015. **175**: p. 249-57.

25. Sharif, H.R., et al., *Current progress in the utilization of native and modified legume proteins as emulsifiers and encapsulants – A review.* Food Hydrocolloids, 2017.

26. Corte-Real, J., et al., *Selective factors governing in vitro beta-carotene bioaccessibility: negative influence of low filtration cutoffs and alterations by emulsifiers and food matrices.* Nutr Res, 2014. **34**(12): p. 1101-10.

27. Institute of Medicine (U.S.). Panel on Macronutrients. and Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes., *Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids*. 2005, Washington, D.C.: National Academies Press. xxv, 1331 p.

28. Bohn, T., *Bioactivity of Carotenoids - Chasms of Knowledge.* Int J Vitam Nutr Res, 2017: p. 1-5.

29. Forman, M.R., et al., *Effect of menstrual cycle phase on the concentration of individual carotenoids in lipoproteins of premenopausal women: a controlled dietary study.* Am J Clin Nutr, 1998. **67**(1): p. 81-7.

30. Goltz, S.R., et al., *Meal triacylglycerol profile modulates postprandial absorption of carotenoids in humans.* Mol Nutr Food Res, 2012. **56**(6): p. 866-77.

31. Van Lieshout, M., et al., *Extraction of carotenoids from feces, enabling the bioavailability of beta-carotene to be studied in Indonesian children.* J Agric Food Chem, 2003. **51**(17): p. 5123-30.

32. Amnuaycheewa, P. and E.G. de Mejia, *Purification, characterisation, and quantification of the soy allergen profilin (Gly m 3) in soy products.* Food Chemistry, 2010. **119**(4): p. 1671-1680.

33. Holzhauser, T., et al., *Soybean (Glycine max) allergy in Europe: Gly m 5 (β-conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy.* J ALLERGY CLIN IMMUNOL, 2009. **123**(2): p. 452-458.

34. Savage, J.H., et al., *The natural history of soy allergy.* J Allergy Clin Immunol, 2010. **125**(3): p. 683-6.

35. Yu, J., et al., *Enzymatic treatment of peanut kernels to reduce allergen levels.* Food Chem, 2011. **127**(3): p. 1014-22.

36. Koppelman, S.J., et al., *Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil–histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen.* Clin Exp Allergy, 2004. **34**(4): p. 583-590.

37. Crevel, R.W., M.A. Kerkhoff, and M.M. Koning, *Allergenicity of refined vegetable oils.* Food Chem Toxicol, 2000. **38**(4): p. 385-93.

38. Gomes-Santos, A.C., et al., *Hydrolyzed whey protein prevents the development of food allergy to beta-lactoglobulin in sensitized mice.* Cell Immunol, 2015. **298**(1-2): p. 47-53.

39. Wal, J.M., *Bovine milk allergenicity.* Ann Allergy Asthma Immunol, 2004. **93**(5 Suppl 3): p. S2-11.

40. Schulmeister, U., et al., *Cloning, expression, and mapping of allergenic determinants of alphaS1-casein, a major cow's milk allergen.* J Immunol, 2009. **182**(11): p. 7019-29.

41. Branum, A.M. and S.L. Lukacs, *Food allergy among U.S. children: trends in prevalence and hospitalizations.* NCHS Data Brief, 2008(10): p. 1-8.

42. Hori, H., et al., *Analysis of the major epitope of the alpha2 chain of bovine type I collagen in children with bovine gelatin allergy.* J Allergy Clin Immunol, 2002. **110**(4): p. 652-7.