J Nutr Health Aging. 2018;

MARKERS OF HYPOXIA AND OXIDATIVE STRESS IN AGING VOLUNTEERS INGESTING LYCOSOMAL FORMULATION OF DARK CHOCOLATE CONTAINING ASTAXANTHIN

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Abstract: *Objective:* To determine if ingestion of lycosome-formulated dark chocolate (DC) containing astaxanthin (ASTX) improves bioavailability of ASTX and affects markers of hypoxia and oxidative stress in aging individuals. *Design:* Randomized, blinded, four-arm, prospective study. *Settings:* Lycotec Ltd, Cambridge, United Kingdom and Institute of Cardiology, Saratov, Russian Federation. *Participants:* 32 healthy individuals aged 60-70 years with confirmed signs of oxidative stress (increased serum levels of oxidized LDL and malonic dialdehyde) randomized into four study groups (8 volunteers each). *Intervention:* Volunteers of first group were given orally 10 gr of dark chocolate (DC). Individuals from the second group received 7 mg of astaxanthin (ASTX). Third group of volunteers was supplemented with 10 gr of DC and 7 mg of ASTX ingested simultaneously as two separate formulations. Last group of the individuals was given 10 gr of a lycosomal formulation of DC containing 7 mg of co-crystalized ASTX (L-DC-ASTX), a newly developed highly bioavailable nutraceutical composition of DC containing 2 groups of antioxidants (cocoa flavanols and ASTX). All formulations were given orally, once daily for a month. *Measurements:* Serum ASTX was measured by high-performance liquid chromatography. Nitric oxide, malonic dialdehyde and oxidized LDL were quantified spectrophotometrically. Oxygenation parameters were evaluated by near-infrared spectroscopy. *Results:* One month ingestion of singular formulation of ASTX lead to a 20 fold buildup in serum ASTX level whereas the 4 week ingestion of L-DC-ASTX formulation was accompanied by more prominent accumulation of ASTX in serum (a 40 fold increase over the basal values) at the same daily dose of ASTX. Both antioxidants taken separately decreased serum levels of oxidized LDL and malonic dialdehyde. However effect of L-DC-ASTX formulation was more prominent. ASTX ingested alone caused a borderline increase ($p=0.054$) in serum nitric oxide (NO) levels, whereas DC ingestion lead to small but statistically significant increase in serum NO concentration. Higher values of NO level were seen after co-ingestion of DC and ASTX, especially in case of L-DC-ASTX formulation suggesting additive/synergistic effects of DC and ASTX on nitric oxide production. These changes were in agreement with the increase in plasma oxygen transport and tissue oxygen saturation seen in the volunteers supplemented with L-DC-ASTX formulation. *Conclusion:* The nutraceutical formulation of DC and ASTX with an enhanced bioavailability of ASTX can be efficiently used for the correction of oxidative status in aging individuals.

Key words: Dark chocolate, astaxanthin, oxidized LDL, nitric oxide.

Introduction

Ageing is often associated with oxidative stress and signs of inflammation reflecting antioxidant status of individuals and their life style choices (diet and physical activity). Recent epidemiological studies establish the link between oxidative stress, cardiovascular health and carotenoid consumption (1-3). It is believed, that carotenoid supplementation may be highly effective in the reduction of cardiovascular disease in general population due to remarkable antioxidant properties of carotenoids (4-6). Astaxanthin (ASTX) is a red hydrophobic keto-carotenoid pigment representing the terpene and xanthophyll classes of organic compounds with an extremely high antioxidant potential. It is synthesized by microalgae as well as some bacteria, yeast and fungi (7). Microalgae ingestion leads to the appearance of ASTX in various marine species such as shellfish, salmon and trout. Humans cannot synthetize ASTX and acquire this carotenoid from sea food products or dietary supplements (8). ASTX is a very powerful antioxidant approved by the FDA as a nutraceutical in 1999, whose anti-radical properties are determined by conjugated double bonds in its polyene backbone and polar ionic rings. Antioxidant activity of ASTX exceeds the anti-radical properties of vitamin C by 65 times and the anti-radical potential of other carotenoids, such as lutein, canthaxanthin, and β-carotene by 10 times (9). ASTX also provides more effective protection from free radicals when compared with vitamin E and other tocopherols and tocotrienols (10).

The high anti-oxidant potential of ASTX translates into a number of health benefits witnessed in different experimental and clinical studies. These include anti-cancer (11), anti-obesity (12), anti-diabetic (13), anti-inflammatory (11), and cardioprotective (14) effects as well as pieces of evidence describing its positive effects on skin, immune and

Received April 13, 2018
Accepted for publication April 13, 2018

hematopoietic (15-16) functions.

There are three isomeric forms of ASTX (3S,3S'; 3R,3S' and 3R,3R') reflecting the orientation of hydroxyl groups at the third carbon atom position (9, 10). However regardless of isomer identity all ASTX analogs are amphiphilic molecules capable of interactions with phospholipids with further formation of ASTX-phospholipid and ASTX-polar lipid complexes (18). Like many other lipophilic carotenoids, synthetic and naturally derived isomers of ASTX have a similarly low intestinal bioavailability rate (19). However, intake of ASTX with dietary fat as well as ingestion of microencapsulated formulations of ASTX enhances its bioavailability and biological effects (20). Nutraceutical nano-delivery formulations of ASTX with an increased bioavailability are recently proposed (21).

Carotenoid delivery technologies, in particular lycosome delivery technology (22, 23), open new perspectives for functional and medicinal food development. It was shown in our previous work that carotenoids (lycopene and ASTX) and phospholipids (phosphatidylcholine) can form microparticles (lycosomes) with an enhanced intestinal absorption rate and resistance to gastro-intestinal enzymes. Moreover carotenoid/phospholipids lycosome particles may be loaded with different "cargo" molecules which may include hydrophobic peptides, polyphenols and others (24-27). Recently we reported a new lycosome-formulated dark chocolate nutraceutical formulation containing lycopene (25). This formulation has a better effect on blood pressure and lipid profile in volunteers with mild hyperlipidemia when compared with regular dark chocolate (25).

In the recent paper we report another nutraceutical formulation of dark chocolate which contains ASTX-based lycosomes. The rationale behind ASTX use was based on the fact that ASTX displays higher radical-quenching capacity than lycopene (9, 28). Moreover, lycopene and ASTX have a great degree of similarity in their physico-chemical properties and their ability to interact with phospholipids, another integral part of lycosome particles. Therefore ASTX-based lycosomes display the same rate of stability and structural integrity as lycopene-based microparticles.

Here we show, that combining two powerful antioxidants (ASTX and dark chocolate flavanols) in one nutraceutical lycosomal product results in an enhanced bioavailability of ASTX and provide better antioxidant protection of aging individuals with oxidative stress.

Materials and methods

The study was initiated and conducted by Lycotec Ltd (Cambridge, UK) at its facilities in Cambridge, UK, and at the Institute of Cardiology, the Ministry of Health of the Russian Federation (Saratov, Russian Federation). The study was designed as a part of a bigger multi-arm trial under a protocol approved by the local Ethics Committee and registered (ACTRN12613000966796). All volunteers were informed of the purpose of the study as well as its outcomes and signed a written consent form regarding their participation. All volunteers underwent physical and laboratory examinations, were asked about their medical history and socio-economic background and were fully informed about study objectives.

Subjects and inclusion/exclusion criteria

From the selected pool of volunteers, four randomized groups from the total of 32 people were formed (Table 1). Eight volunteers were not able to complete the trial due to intolerance of cocoa products or for other reasons not related to health matters. Dropouts were replaced with other eligible pre-selected volunteers.

Inclusion criteria were as follows: Caucasian males or females aged from 60 to 70 years; signed consent form; no anti-hypertensive, lipid-lowering, or anti-diabetic drugs; serum-positivity for markers of inflammation and oxidation such as oxidized Low Density Lipoprotein (LDL-Px ELISA) ≥100 ELISA Units, Inflammatory Oxidative Damage (IOD) ≥100 μM of malonic dialdehyde as well as willingness and ability to comply with the protocol for the duration of the study. Exclusion criteria were: severe medical conditions affecting outcomes of the study (myocardial infarction, stroke, diabetes mellitus, hepatitis, cancer, renal failure, pancreatitis, epilepsy, tuberculosis, HIV, etc.); inability to comply with study protocol and sign written consent; participation in other clinical trials; intolerance of cocoa products or special diets.

All volunteers were asked to abstain from consumption of cocoa-containing and seafood products for 10 days before beginning the study and during the study apart from the samples received in the study. After completion of the run-in period patients were given the trial products.

Study Groups

The duration of the study for each group was 4 weeks. All volunteers underwent medical evaluation and laboratory investigation on day "0" and at the end of the 4th week of the trial. Volunteers were randomly divided into 4 major groups. 8 individuals from the first group (dark chocolate alone group) ingested once daily single (10g) bar of dark chocolate. 8 volunteers from the second group (ASTX alone group) ingested once daily a pill containing 7 mg of ASTX. The third group of volunteers (8 individuals) ingested once daily a dark chocolate bar and a 7 mg of ASTX capsule taken as two separate formulations (concomitant intake of dark chocolate and ASTX group). Volunteers from the fourth group of the study (Esthechoc group, 8 individuals) were asked to ingest once daily a 10 g Esthechoc dark chocolate bar containing 7 mg of ASTX. All study products were taken in the morning after breakfast.

Volunteers in the first group and in the fourth group were double-blinded and were not aware whether they were taking control or ESTHECHOC chocolate.

Blood Collection. Blood was collected in the morning after overnight fast from arm veins of the patients. The serum was separated from the rest of the clotted mass by centrifugation, then aliquots were stored at –80°C prior to analysis.

Study products

Dark chocolate. Dark chocolate bars (10 g) with 72% cocoa from Master Martini (Milan, Italy) were used in all groups of the study. The chocolate was melted, treated, and tempered in precisely the same way for all groups of the study regardless of the addition of ASTX. Nutritional parameters are available from the manufacturer.

Haematococcus microlgae astaxanthin (ASTX) was purchased from Valensa, USA.

Lycosome-formulated Dark Chocolate containing co-crystalized ASTX (ESTECHOC) was prepared using lycosome technology (22, 23). Formation of ASTX lycosomes was shown in our preliminary work to provide mutual protection of both cocoa flavanols and the ASTX from gastrointestinal enzymes and increase their intestinal absorption rate.

Astaxanthin Measurements in Serum

ASTX concentration in serum specimens was measured by HPLC-MS protocol as described (29). Standards were purchased from Sigma.

Biochemistry and Inflammatory Markers

Glucose, serum lipids, and nitric oxide (NO) were measured in serum using commercially available analytical kits according to the manufacturers' recommendations (ByoSystems, R&D Systems).

Oxygenation Parameters

As a tissue target for the assessment of oxygen saturation, StO2, or combined level of oxygenated hemoglobin and myoglobin, we used the eminence and forearm muscles of the patients (27). StO2 was analyzed by continuous wavelength near-infrared spectroscopy, NIRS, with wide-gap second-derivative (In Spectra, Hutchinson Technology, MN, USA). The measurements were made at different time points. The recording began following 15 min rest in a supine position before occlusion of the brachial artery. It then continued during stagnant ischemia induced by rapidly inflating the cuff to 50 mm Hg above systolic BP. The ischemia lasted for 3 min, and the recording period lasted for another 5 min after that until StO2 was stabilized.

Then the area under the hyperemic curve, AUC, of the recorded signal for the settling time in the post-occlusion period was calculated as described earlier in % O2/minute.

Inflammatory Oxidative Damage (IOD)

Plasma samples were incubated overnight in 0.05 M PBS acetate buffer (pH 5.6) which would imitate the type of oxidative damage which occurs during the release of lysosomes following neutrophil degranulation. The following morning, the reaction was terminated using trichloroacetic acid. The concentration of the end products such as malonic dialdehyde (MDA) and other possible thiobarbituric acid reactive substances (TBARS) was then measured by colorimetric methods (30) using reagents and kits from Cayman Chemical (MC, USA).

Oxidized LDL (LDL-Px ELISA)

Activity of serum LDL peroxidase proteins, which include IgG with superoxide dismutase activity, was measured as described earlier (31, 32).

Statistics

For the assessment of normally distributed parameters, the Shapiro–Wilk method was used. Student's t-test was then applied both for paired and unpaired samples. Between group differences at one time point were evaluated by the Wilcoxon–Mann–Whitney test (continuous variables) and Fisher's exact test (categorical variables). Data analysis was performed using Stata (College Station, TX) SE, version 12.1. Variants with normal distribution are shown as averages with standard deviations. Highly variable are parameters represented as medians with confidence intervals. All statistical tests were two sided and statistical significance level alpha was set at 0.05 for the analysis.

Results

Randomization

As can be seen from the Table 1, the protocol used in our study provided successful randomization of volunteers among four major groups of the study. There were no significant variations among BMI values and major vital as well as laboratory parameters among the volunteers.

ASTX Serum Levels

As can be seen from Table 2, ingestion of dark chocolate alone (group 1) for a month was not accompanied by any changes in serum ASTX level. However ingestion of 7 mg of ASTX lead to ~ 20 fold increase in the serum ASTX level.

Nearly similar increase took place in volunteers who ingested dark chocolate and ASTX as two separate formulations. Notably, supplementation of volunteers with lycosome formulation of dark chocolate containing ASTX was accompanied with a ~ 40 fold increase in serum ASTX levels.

Plasma and Tissue Oxygen Parameters

As can be seen from Table 3, ingestion of lycosome formulation of dark chocolate containing ASTX had some effect on tissue oxygen saturation and plasma oxygen transport. In particular, consumption of ESTECHOC formulation caused an 18.8% increase in plasma oxygen transport after 4 week

Table 1
Baseline characteristics (Averages with standard deviations)

VARIABLE	Group 1	Group 2	Group 3	Group 4
Number of Patients	8	8	8	8
Males	4	4	4	4
Females	4	4	4	4
Age	66.40±2.55	64.7 ± 1.95	66.3±1.77	65.31±2.31
Light/Moderate Smokers	1	2	1	2
Body Mass Index	24.41 ± 1.33	23.72±1.55	23.11±1.72	23.55±1.77
AST in U/L	35.22 ± 1.77	38.21 ± 2.33	37.54±1.44	35.98±2.54
ALT in U/L	41.32 ± 2.11	35.21± 1.43	34.21±1.75	35.21±2.03
Fasting Glucose in mmol/L	5.5 ± 0.49	6.2 ± 0.61	5.7±0.91	5.6±0.81
Total Cholesterol in mg/Dl	211.13 ± 11.50	209.3 ± 15.77	215.01±14.3	215.43±11.5
Triglycerides in mg/Dl	128 ± 12.6	129 ± 12.9	132±9.54	143.12±13.2
Pulse rate per min	74.8 ± 3.4	78.1 ± 2.54	76.11±3.21	75.21±2.56
Blood Pressure in mm Hg				
Systolic	118.5±5.3	110.8±7.55	112.0±6.77	118.31±5.90
Diastolic	75.7±5.2	75.1±6.0	72.31±4.88	76.35±5.21

Table 2
Serum levels of ASTX (Averages with Standard Deviations)

ASTX nmol/L	Group 1	Group 2	Group 3	Group 4
"0" Time	0.42 ±0.21	0.37 ±0.15	0.47 ±0.11	0.44 ±0.14
After 4 weeks Ingestion	0.39 ±0.14	7.31 ±2.55*	5.22 ±1.89*	17.34 ±3.55*

(*) – P<0.05

ingestion. Notably, ASTX nor dark chocolate alone had impact on that parameter. In contrast, ASTX regardless of formulation used caused a statistically significant increase in tissue oxygen saturation. Ingestion of ASTX alone was accompanied by 21.7% increase in tissue oxygen saturation. Co-ingestion of ASTX and dark chocolate caused a similar increase in those values. However most prominent upregulation was seen after ingestion of lycosome formulation of dark chocolate containing ASTX (increase by 43.9%, P<0.05). Interestingly, there was a small tendency towards upregulation of tissue oxygen saturation with dark chocolate alone which fell blow the limit of statistical significance accepted in our study (P=0.067).

Oxidized LDL

As can be seen from Table 4, ingestion of dark chocolate alone did not affect the amount of oxidized LDL in the serum of volunteers. However, intake of ASTX as a single formulation caused a significant decrease in oxidized LDL level on oxidized LDL level (reduction of medians by 55.4%). Co-ingestion of dark chocolate and ASTX caused even more prominent decrease in oxidized LDL (decline in medians by 65.02%). Similar degree of LDL reduction was seen after ingestion of lycosome-formulated dark chocolate containing ASTX.

Inflammatory Oxidative Damage (IOD)

There were significant changes in IOD values, as estimated by measurements of malonic dialdehyde (MDA), (Table 4). DC alone did not affect MDA concentration in the serum of volunteers. However, ASTX alone reduced oxidized LDL levels by 52.7%. Similar degree of reduction was seen after co-ingestion of ASTX and dark chocolate. Remarkably, when the ESTECHOC formulation of DC was ingested the inhibitory effect on IOD was significantly stronger than all other groups (reduction of medians by 88.2%).

Nitric Oxide. Both nutraceuticals – dark chocolate and ASTX taken alone caused some increase of serum NO levels. In case of dark chocolate that increase averaged 11% over control level. Less significant increase (P=0.54) was seen in the AST

Table 3
Plasma and tissue oxygen parameters (Averages with Standard Deviations)

GROUPS	Plasma Oxygen Transport		Tissue Oxygen Saturation	
	Pretreatment	4th week	Pretreatment	4th week
Group 1. Dark Chocolate (DC)	685.12 + 31.3	654.6 + 48.0	39.2 + 2.54	42.4 + 3.91
Group 2. ASTX alone	663.21 +27.4	684.8 + 35.5	37.20±1.65	45.3±2.33*
Group 3. Co-ingestion ASTX+DC	683.21±23.44	690.45±34.21	40.20±2.24	46.56±3.01*
Group 4. Lycosome Formulation ASTX and DC	674.25±25.87	801.32±42.12*	37.56±1.99	54.07±3.21*

(*) – P<0.05

Table 4
Oxidized LDL and IOD values (Median Values with 5% and 95% CIs)

GROUPS	LDL Px (ELISAx1000)		IOD (MDA μM)	
	Pretreatment	4th week	Pretreatment	4th week
Group 1. Dark Chocolate (DC)	139.5 (203.6/98.9)	153.5 (216.6/101.4)	116.4 (148.3/89.3)	104.5 (128.1/73.9)
Group 2. ASTX alone	158.4 (189.5/127.2)	70.67 (96.8/44.4)*	125.71 (139.4/111.4)	59.4 (83.3/43.1)*
Group 3. Co-ingestion ASTX+DC	138.4 (179.0/110.4	48.4 (63.8/37.1)*	129.4 (140.8/114.6)	62.5 (82.5/42.5)*
Group 4. Lycosome Formulation ASTX and DC	130.3 (155.3/93/2)	51.5 (71.8/42.8)*	130.3 (164.6/112.3)*	15.3 (37.2/8.7)*

(*) – P<0.05

alone group (Table 5).

Co-ingestion of dark chocolate and ASTX was accompanied by some additive effect of nutraceuticals on serum NO level, which was upregulated at the end point of the study by 14.4%. However, lycosome formulation of dark chocolate with ASTX lead to much higher spike in serum NO level approximating 31.1% over control suggesting thereby occurrence of synergistic effect of dark chocolate and ASTX on nitric oxide production in volunteers.

Table 5
Serum Nitric Oxide Levels (Averages with Standard Deviations, M±m)

GROUPS	Nitric Oxide μM	
	Pretreatment	4th week
Group 1. Dark Chocolate (DC)	25.14 + 0.77	27.90 + 0.95*
Group 2. ASTX alone	25.65 + 0.63	26.79±0.71
Group 3. Co-ingestion ASTX+DC	26.16±0.93	29.94±1.11*
Group 4. Lycosome Formulation ASTX and DC	25.31±1.11	33.20±1.44*

(*) – P<0.05

Discussion

Oxidative stress originates from the inability of the human body to detoxify free radicals due to their overproduction and the absolute or relative deficiency of antioxidants. It has been proven by many researchers that oxidative stress is a key element during the ageing process and plays a substantial role in the pathogenesis of many human diseases such as cardiovascular disease, diabetes and cancer (33) as well as respiratory and neurological disorders (34, 35). Although there has been an exponential increase in the number scientific reports implicating oxidative stress in ageing and the pathogenesis of many other diseases, there have been no cohesive recommendations or approved medications for management and / or treatment of oxidative stress. Moreover, multiple attempts to promote habitual intake of antioxidant-containing food have failed to improve the antioxidant status of volunteers in many clinical studies due to the low intestinal absorption rate of naturally occurring antioxidants and their susceptibility to oxidation in the gut environment (36).

In this paper we analyze the effects of two of the most powerful naturally occurring antioxidant products – dark chocolate, rich in cocoa flavanols, and astaxanthin (ASTX) ingested separately or simultaneously as two separate formulation. Moreover, microencapsulated lycosomal formulation of dark chocolate and ASTX was tested. Theoretically, the combinatory formulations of antioxidants may hold a significant promise for pharmacotherapy of oxidative stress and related disorders by endorsing the additive effects of antioxidants on markers of biological oxidation especially if the antioxidants target different oxidative pathways. `Such additive effect of dark chocolate and ASTX ingested simultaneously as two separate formulations was seen

in our study on values of oxidized LDL, nitric oxide level and to some extend on parameters of oxygenation. All changes in oxidative parameters of volunteers observed in our study are highly interrelated singularities. As we have shown above, changes in oxidized LDL clearly follow the pattern of malonic dialdehyde changes in the groups of the study suggesting thereby a pivotal role of peroxides in LDL oxidation. There is a certain degree of agreement between changes of oxygenation parameters and nitric oxide levels in the study groups which reflects a well-known relationship between NO production and peripheral hemodynamics.

However, most important finding reported in this paper is related to the fact that ingestion of lycosomal formulation of dark chocolate containing ASTX significantly improves bioavailability rate of ASTX. As we have shown, one month ingestion of singular formulation of ASTX leads to a 20 fold buildup in serum ASTX level whereas, the 4 week ingestion of lycosomal formulation of ASTX is accompanied by more prominent accumulation of ASTX in serum which approximates a 40 fold increase over the basal values at the same daily dose. As a result, the lycosomal formulation of dark chocolate with ASTX was more beneficial in the correction of parameters of oxidative stress in aging volunteers.

ASTX is known to be a poorly absorbed chemical compound, whose intestinal absorption rate can be significantly improved by co-ingestion with dietary fat (19, 20). Better bioavailability of ASTX ingested as an integral component of lycosome particles might be explained by better degree of ASTX protection from gastro-intestinal enzymes as well as by the presence of cocoa butter lipids in the dark chocolate crystals. Therefore ingestion of lycosomal formulation of dark chocolate and ASTX goes far beyond mere additive effects of two co-ingested nutraceuticals and finalizes in the improved bioavailability of ASTX as well as appearance of possible synergistic effects of dark chocolate and ASTX in oxidative stress. This was clearly seen in the pattern of nitric oxide level changes. It has to be clarified that changes in nitric oxide production is a main physiological mechanism mediating effects of cocoa polyphenols on the cardiovascular system (37) and it was a highly expected outcome in our study. The novelty comes from the fact that the effects of dark chocolate on nitric oxide production can be potentiated by co-ingestion of ASTX and synergized in the case of ingestion of lycosomal formulation of dark chocolate and ASTX.

We should state that our study had certain limitations. First of all, increased bioavailability of ASTX seen in volunteers after ingestion of lycosome formulation of dark chocolate may be accompanied by parallel changes in bioavailability of cocoa flavanols, which are another integral part of lycosome micro-particles. Besides ASTX, lycosome formulated dark chocolate contains a number of powerful antioxidants originating from Theobroma cacao beans - catechins, proanthocyanidins, anthocyanins, methylxantines and mono phenolic acids (37) whose bioavailability may potentially affect end points of the study and needs be monitored in future work. Secondly, it would have been also useful to evaluate other parameters of biological oxidation and markers of aging in further studies. And finally, a larger number of volunteers and longer supplementation periods are required to re-confirm and extend the findings reported above.

Ethical standard: The study was conducted accordingly to the Ethical Principles for Medical Research involving Human Subjected stated by Helsinki Declaration and current government regulations for clinical trials in both United Kingdom and Russian Federation.

Conflicts of interest: All authors declare no conflict of interest involved.

Acknowledgments: Ivan Petyaev and Yuriy Bashmakov were involved in planning, conducting the study, analyzing the results and writing the manuscript. Natalya Chalyk and Victor Klochkov performed most of the clinical work. Nigel Kyle, Dmitry Pristensky and Marina Chernyshova were responsible for analytical assays. All authors contributed to the writing of the manuscript.

References

1. M. D. Shardell, D. E. Alley, G. E. Hicks et al., "Low-serum carotenoid concentrations and carotenoid interactions predict mortality in US adults: the Third National Health and Nutrition Examination Survey," Nutrition Research, vol. 31, no. 3, pp. 178–189, 2011.
2. Y. Ito, M. Kurata, K. Suzuki, N. Hamajima, H. Hishida, and K. Aoki, "Cardiovascular disease mortality and serum carotenoid levels: a Japanese population-based follow-up study," Journal of Epidemiology, vol. 16, no. 4, pp. 154–160, 2006.
3. X. Li and J. Xu, "Dietary and circulating lycopene and stroke risk: a metaanalysis of prospective studies," Scientific Reports, vol. 4, p. 5031, 2014.
4. M. S. Donaldson, "A carotenoid health index based on plasma carotenoids and health outcomes," Nutrients, vol. 3, no. 12, pp. 1003–1022, 2011.
5. Petyaev IM. Lycopene Deficiency in Ageing and Cardiovascular Disease. Oxid Med Cell Longev. 2016;2016:3218605.
6. Bahonar A, Saadatnia M, Khorvash F, Maracy M, Khosravi A. Carotenoids as Potential Antioxidant Agents in Stroke Prevention: A Systematic Review. Int J Prev Med. 2017 Sep 14;8:70.
7. Henríquez V, Escobar C, Galarza J, Gimpel J. Carotenoids in Microalgae. Subcell Biochem. 2016;79:219-37.
8. Alcaíno J, Baeza M, Cifuentes V. Carotenoid Distribution in Nature. Subcell Biochem. 2016;79:3-33.
9. Visioli F, Artaria C. Astaxanthin in cardiovascular health and disease: mechanisms of action, therapeutic merits, and knowledge gaps. Food Funct. 2017 Jan 25;8(1):39-63.
10. Gammone MA, Riccioni G, D'Orazio N. Marine Carotenoids against Oxidative Stress: Effects on Human Health. Mar Drugs. 2015 Sep 30;13(10):6226-46.
11. Kang H, Kim H. Astaxanthin and β-carotene in Helicobacter pylori-induced Gastric Inflammation: A Mini-review on Action Mechanisms. J Cancer Prev. 2017 Jun;22(2):57-61.
12. Kitade H, Chen G, Ni Y, Ota T. Nonalcoholic Fatty Liver Disease and Insulin Resistance: New Insights and Potential New Treatments. Nutrients. 2017 Apr 14;9(4).
13. Bonet ML, Canas JA, Ribot J, Palou A. Carotenoids in Adipose Tissue Biology and Obesity. Subcell Biochem. 2016;79:377-414.
14. Fassett RG, Coombes JS. Astaxanthin, oxidative stress, inflammation and cardiovascular disease. Future Cardiol. 2009 Jul;5(4):333-42.
15. Anunciato TP, da Rocha Filho PA. Carotenoids and polyphenols in nutricosmetics, nutraceuticals, and cosmeceuticals. J Cosmet Dermatol. 2012 Mar;11(1):51-4.
16. Chew BP, Park JS. Carotenoid action on the immune response. J Nutr. 2004 Jan;134(1):257S-261S
17. Jackson H, Braun CL, Ernst H. The chemistry of novel xanthophyll carotenoids. Am J Cardiol. 2008 May 22;101(10A):50D-57D.
18. Polyakov NE, Magyar A, Kispert LD. Photochemical and optical properties of water-soluble xanthophyll antioxidants: aggregation vs complexation. J Phys Chem B. 2013 Sep 5;117(35):10173-82
19. Begum H, Yusoff FM, Banerjee S, Khatoon H, Shariff M. Availability and Utilization of Pigments from Microalgae. Crit Rev Food Sci Nutr. 2016 Oct 2;56(13):2209-22.
20. Anarjan N, Nehdi IA, Sbihi HM, Al-Resayes SI, Malmiri HJ, Tan CP. Preparation of astaxanthin nanodispersions using gelatin-based stabilizer systems. Molecules. 2014 Sep 10;19(9):14257-65.
21. Polyakov NE, Kispert LD. Water soluble biocompatible vesicles based on polysaccharides and oligosaccharides inclusion complexes for carotenoid delivery. Carbohydr Polym. 2015 Sep 5;128:207-19.
22. Petyaev IM. Lycosome Technology: Advances and Perspectives. American Journal of Food Science and Nutrition. 2016, Vol. 3, No. 1, pp. 18-23.
23. Petyaev I. 2012. Carotenoid particles and uses thereof. Patent WO 2012104576 A2. Aug 9, 2012.
24. Petyaev IM. Improvement of hepatic bioavailability as a new step for the future of

statin. Arch Med Sci. 2015 Apr 25;11(2):406-10.
25. Petyaev IM, Dovgalevsky PY, Chalyk NE, Klochkov V, Kyle NH. Reduction in blood pressure and serum lipids by lycosome formulation of dark chocolate and lycopene in prehypertension. Food Sci Nutr. 2014 Nov;2(6):744-50.
26. Bashmakov YK, Assaad-Khalil SH, Abou Seif M, Udumyan R, Megallaa M, Rohoma KH, Zeitoun M, Petyaev IM. Resveratrol promotes foot ulcer size reduction in type 2 diabetes patients. ISRN Endocrinol. 2014 Feb 20;2014:816307.
27. Petyaev IM, Dovgalevsky PY, Klochkov VA, Chalyk NE, Kyle N. Whey protein lycosome formulation improves vascular functions and plasma lipids with reduction of markers of inflammation and oxidative stress in prehypertension. ScientificWorldJournal. 2012;2012:269476.
28. Petyaev IM. Lycopene Deficiency in Ageing and Cardiovascular Disease. Oxid Med Cell Longev. 2016;2016:3218605.
29. Miyazawa T, Nakagawa K, Kimura F, Satoh A, Miyazawa T. Plasma carotenoid concentrations before and after supplementation with astaxanthin in middle-aged and senior subjects. Biosci Biotechnol Biochem. 2011;75(9):1856-8.
30. Moore K, Roberts LJ. Measurement of lipid peroxidation. Free Radic Res. 1998 Jun;28(6):659-71.
31. Petyaev I, Mitchinson MMJ, Hunt JV, Coussons PJ. Superoxide dismutase activity of antibodies purified from the human arteries andatherosclerotic lesions. Biochemical Society Transactions. 1998;26(1):p. S43.
32. Petyaev IM, Coussons PJ. Superoxide Dismutase: Recent Advances and Clinical Applications. Paris, France: Editions Mel; 1999. Superoxide dismutase activity of antibodies purified from human atherosclerotic lesions; pp. 51–54.
33. Mao X, Gu C, Chen D, Yu B, He J. Oxidative stress-induced diseases and tea polyphenols. Oncotarget. 2017 Sep 14;8(46):81649-81661.
34. Sundar IK, Sellix MT, Rahman I. Redox regulation of circadian molecular clock in chronic airway diseases. Free Radic Biol Med. 2017 Oct 31. pii: S0891-5849(17)31165-6.
35. Nakamura T, Lipton SA. SNO'-Storms Compromise Protein Activity and Mitochondrial Metabolism in Neurodegenerative Disorders. Trends Endocrinol Metab. 2017 Oct 30. pii: S1043-2760(17)30134-0.
36. Espín JC, González-Sarrías A, Tomás-Barberán FA. The gut microbiota: A key factor in the therapeutic effects of (poly)phenols. Biochem Pharmacol. 2017 Sep 1;139:82-93.
37. Petyaev IM, Bashmakov YK. Dark Chocolate: Opportunity for an Alliance between Medical Science and the Food Industry? Front Nutr. 2017 Sep 26;4:43.