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Original article

Supplementation of patients with sickle cell disease with astaxanthin increases plasma- and erythrocyte-astaxanthin and may improve the hemolytic component of the disease

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ABSTRACT

Aim & background: Sickle cell disease (SCD) is characterized by hemolytic and vaso-occlusive components. Astaxanthin is a carotenoid of marine origin, without pro-oxidant properties.
Methods: In this open label pilot study, we investigated whether orally administered astaxanthin incorporates into erythrocytes (RBC) of SCD patients and studied the effect on hematological and clinical chemical parameters. Ten SCD patients (6–52 years) in Sint Maarten received 8–12 mg astaxanthin during 3 months.
Results: Baseline plasma- (33 nmol/L) and RBC- (11 nmol/L packed RBC) astaxanthin increased to 225, 174, 167 nmol/L (plasma) and 149, 100, 71 nmol/L packed RBC at 1–3 months, respectively. Reticulocytes decreased from baseline and 2 months (9.5 and 8.8%) to 3 months (5.6%), MCV from 2 to 3 months (88–86 fL), MCH from baseline to 3 months (30–28 pg) and RDW from baseline and 2 months (19.2 and 19.0%) to 3 months (16.7%). Plasma arginine decreased from 2 to 3 months (46.6–39.4 μmol/L). Asymmetric dimethylarginine (ADMA) did not change. Reticulocytes at baseline correlated with relative changes in reticulocytes from baseline to 3 months. Relative changes in reticulocytes correlated with relative changes in RBC, RDW, LDH, ALAT, but not hematocrit, within the same period.
Conclusion: Astaxanthin incorporates into SCD RBC and may favorably affect the hemolytic component. A larger randomized controlled trial is indicated, using similar or higher dose, preferably during more than 3 months, concomitant with (other) low dose antioxidants (vitamin E, beta-carotene, vitamin C, folic acid), minerals (zinc, if necessary, selenium), arginine, fish oil and vitamin D.
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1. Introduction

Sickle cell disease (SCD) is a heterogenous disorder that is mechanistically characterized by hemolytic and vaso-occlusive components. The latter gives rise to cumulative ischemic organ damage[1] that may occasionally precipitate to painful vaso-occlusive crises; all jointly contributing to diminished quality of life and early death.[2] The hemolytic component may find an important trigger in the generation of reactive oxygen species (ROS) by HbS close to the lipid peroxidation-sensitive erythrocyte (RBC) membrane,[3,4] ending up in hemolysis.[3] The vaso-occlusive component may be largely driven by the aforementioned hemolytic component. The resulting enhanced sickle RBC turnover in HbSS patients (RBC half-life 5–10 days[2]) gives rise to a very young

Abbreviations: ADMA, asymmetric dimethylarginine; ALAT, alanine aminotransferase; HbS, sickle-cell hemoglobin; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; NO, nitric oxide; RBC, red blood cell; RDW, RBC distribution width; ROS, reactive oxygen species; SCD, sickle cell disease.

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RBC population with a tendency to adhere to activated vascular endothelium,[5] notably in the post-capillary venules.[6] While sickle RBCs activate the vascular endothelium, the activated endothelium expresses adhesion molecules, providing a pro-adhesive surface for young RBCs and leukocytes.[7] The hemolytic component also negatively affects the hemodynamic stability by reducing nitric oxide (NO) availability[8] through different mechanisms, including NO scavenging by cell-free hemoglobin,[9] increased circulating arginase activity,[10,11] low levels of circulating arginine (NO precursor)[12] and inhibition of NO-synthase through the presence of increased plasma asymmetric dimethylarginine (ADMA).[13,14] The elevated plasma ADMA levels in SCD patients relate to the hemolytic component[13,15] and may derive from proteolysis following hemolytic stress.[16,17]

Targeting the hemolytic component, and notably oxidative stress, by amelioration of the devastating vaso-occlusive component seems a logical intervention strategy for SCD. Oxidative stress may indeed aggravate the symptoms of SCD[18] and may be counteracted by naturally occurring low-toxicity nutrients.[19,20] Probably due to the increased and constant need to neutralize the oxidative stress, SCD patients exhibit important depletions of various antioxidants,[21] including retinol, alpha-tocopherol, and β-carotene, together with a reduced activity of RBC Cu/Zn-superoxide dismutase and Se-glutathione peroxidase.[22] Various trials with naturally occurring antioxidants with promising outcomes have been reported, including those with vitamin E,[23] curcuminoids,[24] aged garlic extract,[25] N-acetylcysteine[26] and zinc.[27]

Astaxanthin is a unique carotenoid. The natural form, predominantly of marine origin, is an antioxidant without pro-oxidant properties[28–30] or side-effects after oral intake.[31] It belongs to the xanthophyll family, providing the pink–red color to certain microalgae (i.e. *Haematococcus pluvialis*)[32] and accumulates in various animals higher in the food chain such as flamingoes, salmon, shrimps and crayfish.[33] The astaxanthin molecule spans the phospholipid double layer of cell membranes due to its two polar head groups that are interspaced by a branched carbon atom chain containing 9 conjugated double bonds. Among the carotenoids that have been shown to incorporate into RBCs of healthy subjects, we can find β-carotene,[34,35] lutein[36] and astaxanthin.[37] Astaxanthin has been found to enhance the immune response,[38,39] to decrease oxidative damage-related symptoms[37,40] and has been proven effective in several diseases and conditions, such as Alzheimer's disease,[41] obesity,[39] asthma, enlarged prostate,[42] osteoarthritis and rheumatoid arthritis.[43]

Due to its unique antioxidant properties, we hypothesized that astaxanthin supplementation might ameliorate the hemolytic component of SCD. For this purpose, we performed an open label pilot study with 10 SCD patients in Dutch Sint Maarten (Caribbean Sea; 18.0237°N 63.0458°W). The transatlantic slave trade introduced the sickle gene into the former Dutch Caribbean, giving rise to an estimated heterozygote (HbAS) prevalence of 6.84% in Sint Maarten, 2.65% in Aruba, and 5.03% in Curaçao. It was estimated that Dutch Sint Maarten harbors 122 SCD patients (40 HbSS and 82 HbSC) among its 50,300 inhabitants.[44] We investigated the effect of

Table 1
Astaxanthin in plasma and erythrocytes together with hematological and clinical parameters during 3 months oral supplementation of sickle cell patients with astaxanthin.

	Reference values	Sampling point			
		Baseline	1 month	2 months	3 months
Plasma astaxanthin (nmol/L)	–	33.3 (18.5–80.3)[b,c,d]	224.6 (84.3–510.2)[a]	173.9 (75.1–324.3)[a]	166.6 (68.8–281.1)[a]
RBC-astaxanthin (nmol/L packed cells)	–	11.2 (1.2–32.1)[b,c,d]	148.5 (39.5–400.9)[a]	100.3 (22.3–180.4)[a]	71.3 (3.4–131.5)[a]
% Astaxanthin in RBC (with plasma astaxanthin = 100%)	–	36.2 (4.0–149.9)[b]	66.2 (33.2–164.9)[a]	58.7 (29.6–100.1)	51.0 (18.9–80.8)
Hemoglobin (g/dL)	12.0–15.0	8.3 (6.9–10.1)	8.4 (7.0–10.2)	8.7 (8.3–9.7)	8.6 (7.3–11.2)
Hematocrit (%)	37.0–52.0	28.2 (23.6–36.9)	26.1 (21.4–30.3)	27.6 (25.4–30.4)	25.1 (19.3–31.0)
RBC (10^3/μL)	4.20–6.10	2.85 (2.13–4.16)	3.00 (2.13–4.54)	3.10 (2.30–4.43)	3.20 (2.09–4.86)
Reticulocytes (%)	0.5–2.5	9.5 (3.3–17.7)[d]	8.5 (5.0–15.5)	8.8 (3.7–15.7)[d]	5.6 (1.8–10.7)[a,c]
RDW (%)	11.5–14.5	19.2 (14.9–23.3)[d]	19.1 (14.2–22.7)	19.0 (15.2–22.4)[d]	16.7 (12.0–20.0)[a,c]
MCV (fL)	80.0–99.0	88.4 (74.9–102.0)	85.8 (72.1–103.0)	87.8 (68.6–106.0)[d]	85.5 (69.1–99.6)[c]
MCH (pg)	27.0–31.0	29.8 (24.0–35.3)[d]	28.9 (22.5–36.3)	28.9 (21.4–36.4)	28.0 (20.2–35.5)[a]
MCHC (g/dL)	33.0–37.0	33.7 (30.8–35.7)	33.6 (31.2–37.8)	32.8 (30.3–36.9)	32.5 (29.3–35.6)
WBC (10^3/μL)	4.8–10.8	15.2 (4.6–50.6)	10.0 (4.4–20.0)	8.9 (3.6–12.2)	9.4 (3.3–14.7)
Neutrophils (%)	37.0–80.0	41.0 (17.2–60.9)	42.2 (27.6–59.7)	45.3 (29.5–59.0)	44.5 (24.9–65.5)
Lymphocytes (%)	10.0–50.0	43.9 (20.6–75.0)	40.3 (26.4–55.2)	37.0 (24.8–47.5)	40.3 (25.6–63.2)
Monocytes (%)	0.0–12.0	8.6 (2.3–13.7)	10.3 (3.6–19.5)	10.0 (3.8–20.2)	7.5 (2.4–14.9)
Eosinophils (%)	0.0–6.0	4.8 (0.9–14.7)	5.3 (1.3–15.5)	6.2 (0.7–16.8)	6.3 (1.0–21.0)
Basophils (%)	0.0–2.0	1.7 (0.5–4.4)	2.0 (0.5–4.0)	1.7 (0.7–2.6)	1.4 (0.5–3.0)
Platelets (10^3/μL)	150–450	376 (157–538)	399 (167–644)	421 (136–705)	383 (157–659)
MPV (fL)	7.4–10.4	9.0 (7.2–13.3)	8.9 (7.4–11.8)	8.6 (6.3–11.7)	8.4 (7.1–10.8)
LDH (U/L)	336–618	1322 (775–2250)	1247 (743–1845)	1201 (665–1551)	1144 (703–1628)
Total bilirubin (mg/dL)	0.2–1.1	3.19 (1.5–5.9)	3.7 (1.6–7.4)	3.1 (1.2–6.4)	2.9 (1.4–7.8)
Indirect bilirubin (mg/dL)	–	3.1 (1.4–5.8)	3.6 (1.5–7.3)	3.0 (1.1–6.3)	2.8 (1.3–7.7)
Creatinine (mg/dL)	0.7–1.2	0.6 (0.4–1.0)	0.6 (0.3–0.9)	0.6 (0.3–0.9)	0.6 (0.4–0.9)
CRP (mg/dL)	0.0–0.8	1.3 (0.0–3.8)	0.9 (0.0–1.3)	1.3 (0.0–3.0)	3.3 (0.0–9.0)
ALAT (U/L)	50–120	33(20–42)	38 (20–70)	34 (9–63)	41 (23–67)
Arginine (μmol/L)	94.2 ± 25.8*	45.2 (13.2–62.5)	46.0 (21.1–65.3)	46.6 (26.6–65.9)[d]	39.4 (16.4–60.4)[c]
Homoarginine (μmol/L)	1.50 ± 0.52**	1.18 (0.80–1.43)	1.20 (0.81–1.95)	1.18 (0.72–2.00)	1.14 (0.84–1.57)
ADMA (μmol/L)	0.42 ± 0.06*	0.75 (0.59–0.94)	0.76 (0.69–0.88)	0.78 (0.63–1.10)	0.74 (0.64–0.95)
SDMA (μmol/L)	0.47 ± 0.08*	0.55 (0.40–0.90)	0.56 (0.41–0.82)	0.54 (0.42–0.75)	0.53 (0.36–0.76)
ADMA/Arginine × 10^3	3.9–4.8***	20.8 (11.8–61.5)	19.44 (11.41–41.9)	18.26 (10.7–32.2)	21.8 (11.8–58.1)

Data are means (range) for 10 patients. Paired-samples *t*-tests applying Bonferroni correction were used to determine whether there were significant differences within the different measurement points in all the measured parameters. [a], significantly different from baseline ($p < 0.01$); [b], significantly different from 1 month ($p < 0.01$); [c], significantly different from 2 months ($p < 0.01$); [d], significantly different from 3 months ($p < 0.01$). * Data from Ref. 48; ** Data from Ref. 50; *** Data from Ref. 13.
Abbreviations: ADMA, asymmetric dimethylarginine; ALAT, alanine aminotransferase; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RBC, red blood cells; RDW, red blood cell distribution width; SDMA, symmetric dimethylarginine; WBC, white blood cells.

a daily 8–12 mg oral dose during 3 months, on plasma- and RBC-astaxanthin levels (primary goal) and several hematological and clinical chemical parameters (secondary goal), including reticulocyte count, mean corpuscular volume (MCV), RBC distribution width (RDW), lactate dehydrogenase (LDH) and ADMA.

2. Materials and methods

2.1. Study design and study group

In this open label pilot intervention study, we included 10 laboratory-confirmed SCD outpatients (7 adults, 3 children, of which 3 males and 7 females, mean age 31 years, range 6–52 years) from the Sint Maarten Medical Centre, with a mean height of 170 cm (range 155–195 cm), mean weight of 58 kg (range 29–91 kg) and mean body mass index of 21 kg/m^2 (range 16–27 kg/m^2). Exclusion criteria were painful crisis and blood transfusion in the preceding 4 weeks and 4 months respectively, pregnancy or the desire to get pregnant in the following 3 months, lactation, active infections and/or auto-immune inflammatory diseases. Drop-out criterion was blood transfusion during the study. Most of the information was obtained from their medical records. Weight and length were measured on the spot.

All participants received verbal and written explanation of the objectives and procedure of the study and subsequently provided us with written informed consent for being included in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. All procedures involving human subjects were approved by the Sint Maarten Medical Center-Medical Staff Medical Ethical Committee (1A-09-2011, dated September 6, 2011).

2.2. Astaxanthin supplementation

Patients were instructed to take a daily dose of 8–12 mg astaxanthin (soft gel gelatin capsules containing an astaxanthin extract from the alga *H. pluvialis*; Cyanotech) during 3 months. The

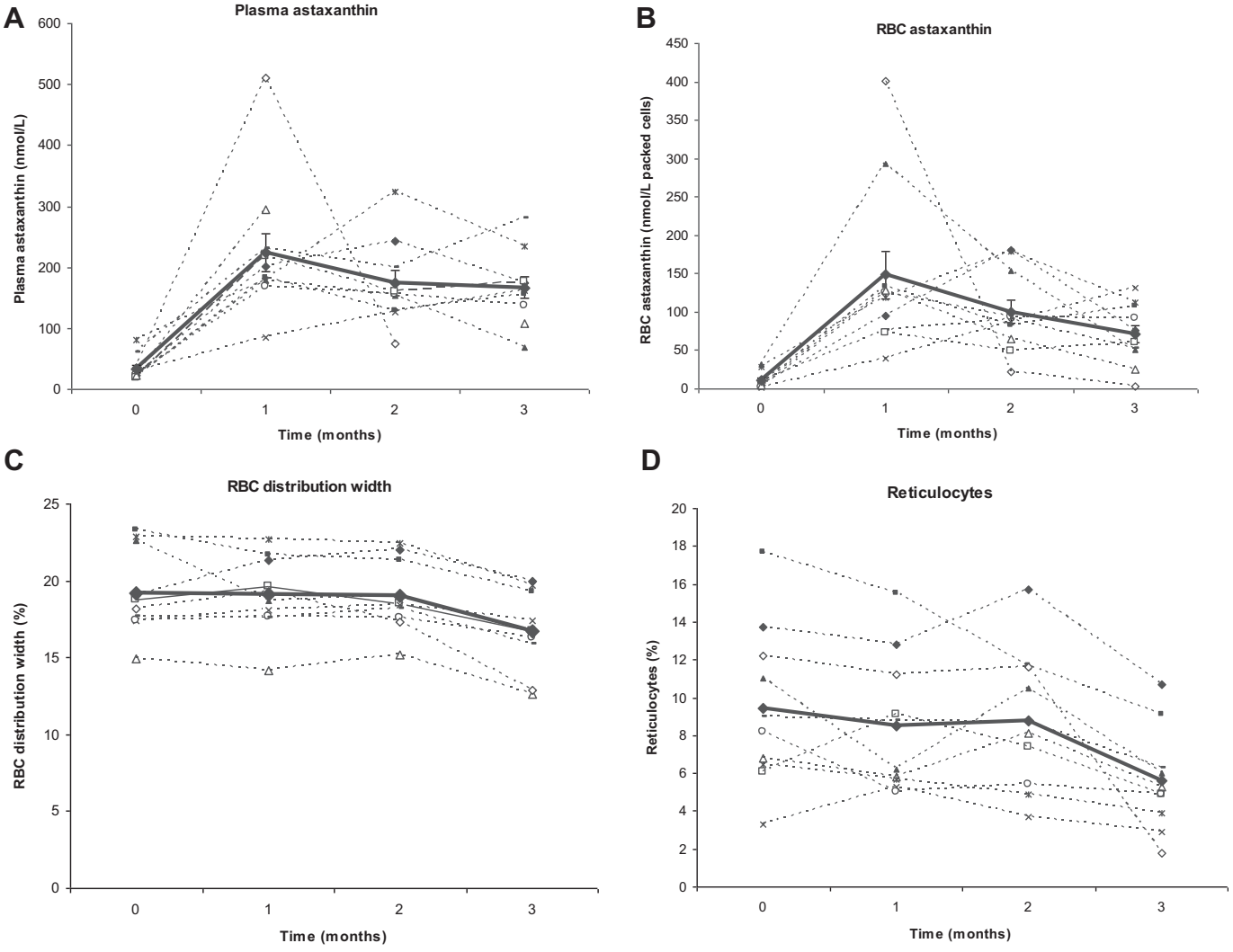

Fig. 1. Courses of plasma astaxanthin (panel A) and RBC-astaxanthin (panel B), red cell distribution width (panel C) and reticulocytes (panel D) of SCD patients during 3 months oral supplementation with astaxanthin. Patients ($n = 10$) received 8 or 12 mg astaxanthin (12 mg/70 kg) daily during 3 months. Each soft gel capsule contained 4 mg astaxanthin, 10 IU vitamin E (d-alpha-tocopherol), 64 μg β-carotene, 40 μg lutein and 72 μg canthaxanthin. Dotted lines represent the courses of individual SCD patients; bold lines represent their means. Both plasma- (panel A) and RBC- (panel B) astaxanthin increased from baseline to 1–3 months supplementation. There were high intra- and inter-individual variations in both plasma- and RBC-astaxanthin concentrations. RDW (panel C) decreased from both baseline and 2 months to 3 months, and reticulocytes (panel D) decreased from 2 to 3 months. Abbreviations: RBC, red blood cells, RDW, red blood cell distribution width.

dose was based on 12 mg astaxanthin/70 kg. Children weighing less than 40 kg took 8 mg astaxanthin/day and adults and children above 40 kg took 12 mg astaxanthin/day. Each soft gel capsule contained 4 mg astaxanthin, 10 IU vitamin E (as d-alpha-tocopherol), 64 μg β-carotene, 40 μg lutein and 72 μg canthaxanthin. The capsules contained glycerol and safflower oil as wetting and filling agents, respectively. The capsules were taken in the morning together with or just after a fat-containing breakfast, as astaxanthin absorption is improved in the presence of lipid based formulations.[45] A compliance intake form was handed to all patients to check daily capsules intake.

2.3. Sample collection and analyses

Blood (3 mL) and EDTA-anticoagulated blood (4 mL) were collected by venipuncture from fasting subjects at baseline and after 1, 2 and 3 months astaxanthin supplementation. To avoid major variations in the plasma- and/or RBC-astaxanthin levels, patients were asked to visit the hospital for blood sampling in the morning, 20–24 h after the last astaxanthin intake.

Serum was separated by centrifuging the blood for 10 min at 1200 g. Measurements of CRP, LDH, bilirubin, creatinine and alanine aminotransferase (ALAT) were performed in Sint Maarten Medical Center (Vitros ® 5600 Integrated System, Johnson & Johnson, Puerto Rico).

EDTA-anticoagulated whole blood was used for a complete blood cell count (RBCs, white blood cells and platelets) and the measurements of hemoglobin, hematocrit and reticulocytes in the Sint Maarten Medical Center (Cell-Dyn ® 3200, Oduber Agencies (Abbott Diagnostics), Curaçao). The remaining EDTA-blood was centrifuged for 10 min at 1000 g for the separation of plasma and RBCs in a cooled centrifuge (4 °C). 200 μL of EDTA-plasma were transferred into a teflon-sealable Sovirel tube containing 2.75 mL of an antioxidant solution (containing EDTA, ascorbic acid, pyrogallol and butylated hydroxytoluene in methanol/water) for the preservation of carotenoids. The remaining EDTA-plasma was subsequently divided in equal portions of about 250 μL and pipetted into 2.5 mL round plastic tubes.

After both plasma and buffy coat were removed, RBCs were washed three times with 0.9% NaCl to prepare packed cells. After washing, the cell mass was suspended in 1 mL phosphate-buffered saline (pH = 7.4). From this suspension, 500 μL were transferred into a teflon-sealable Sovirel tube containing 2.75 mL of an antioxidant mix for the preservation of carotenoids. Total cell counts of the washed RBC suspensions were also performed.

All tubes were frozen at −20 °C until transport and analyses in The Netherlands. Transport to The Netherlands was done in dry ice. Plasma- and RBC-astaxanthin were determined with HPLC/VIS in the University Medical Center Groningen (UMCG; The Netherlands), using previously described procedures.[46,47] For the calculation of RBC-astaxanthin, we corrected the astaxanthin concentration for the hematocrit to obtain the concentration per packed cells.

Plasma arginine, homoarginine, ADMA and symmetric dimethylarginine (SDMA) were determined with reversed-phase HPLC with fluorescence detection in the VU Medical Center Amsterdam (VUmc; The Netherlands) using previously described procedures.[48,49]

2.4. Statistics

Statistical analyses were performed with PASW version 18.0 (SPSS Inc, Chicago, IL). Data were analyzed with paired-samples *t*-tests applying Bonferroni correction for multiple time points to determine whether there were significant longitudinal changes in each of the parameters. Bivariate correlations using Spearman's correlation coefficient were performed between reticulocytes (%) at baseline and the relative change in reticulocytes (%) between 3 months and baseline, and also between the relative change in reticulocytes (%) between 3 months and baseline and the changes in the same period of the other parameters. Linear regression was used to model the relationship between the aforementioned variables, for the calculation of both the slope and intercept of the linear equation from the observed correlations.

3. Results

All patients had been diagnosed with homozygous SCD (HbSS) in Sint Maarten. The occasionally low MCV suggests that at least 7 of them might have concomitant alpha-thalassemia or (less probably) HbSβ°. Their hemoglobin profiles, as established with HPLC at baseline, did not reveal any HbA. We found that the supplement was well tolerated. There were no signs or complaints of side-effects and many of the patients reported spontaneously that they 'felt much better' during the study.

3.1. Astaxanthin supplementation increased plasma- and RBC-astaxanthin levels

Both plasma- and RBC-astaxanthin increased from baseline (plasma mean: 33 nmol/L; RBC mean: 11 nmol/L packed RBC, respectively) to 1–3 months (plasma means: 225, 174, 167 nmol/L: RBC means: 149, 100, 71 nmol/L packed RBC) (Table 1). Fig. 1 shows the courses of the mean plasma- (panel A) and RBC- (panel B) astaxanthin, together with the individual courses across the 3 months of supplementation.

3.2. Astaxanthin supplementation decreased reticulocytes, RDW, MCV and MCH

Hematological parameters are shown in Table 1. Reticulocyte percentages decreased from baseline to 3 months (9.5–5.6%) and from 2 to 3 months (8.8–5.6%). Concomitantly, also the RDW decreased from baseline to 3 months (19.2–16.7%) and from and 2–3 months (19.0–16.7%). The MCV decreased from 2 to 3 months (87.8–85.5 fL), and the MCH decreased from baseline to 3 months (29.8–28.0 pg). Fig. 1 shows the courses of the RDW (panel C) and reticulocytes (panel D) across the 3-month astaxanthin supplementation period, together with the individual courses for the 10 patients. No significant differences were found between any of the other measured hematological and clinical chemical parameters across the different sampling points (Table 1).

3.3. Astaxanthin supplementation decreased plasma arginine but did not change homoarginine, ADMA, SDMA and the ADMA/arginine ratio

We found that the plasma arginine concentrations of the SCD patients (Table 1) were lower and ADMA levels higher, than the reference values that have previously been established with the same method (Table 1).[48] Current arginine levels were also lower than those of our own historical controls (for subjects with HbAA (median 67; range 60–88 nmol/L)[13]). On the other hand, the SDMA (Table 1; historical controls HbAA: median 0.33; range 0.33–0.35 nmol/L) and ADMA concentrations (HbAA: median 0.33; range 0.32–0.35 nmol/L) were higher in the current SCD study group. During astaxanthin supplementation, arginine levels decreased from 2 to 3 months (46.6–39.4 nmol/L), but no changes were found in either homoarginine, ADMA, SDMA or the ADMA/arginine ratio (Table 1).

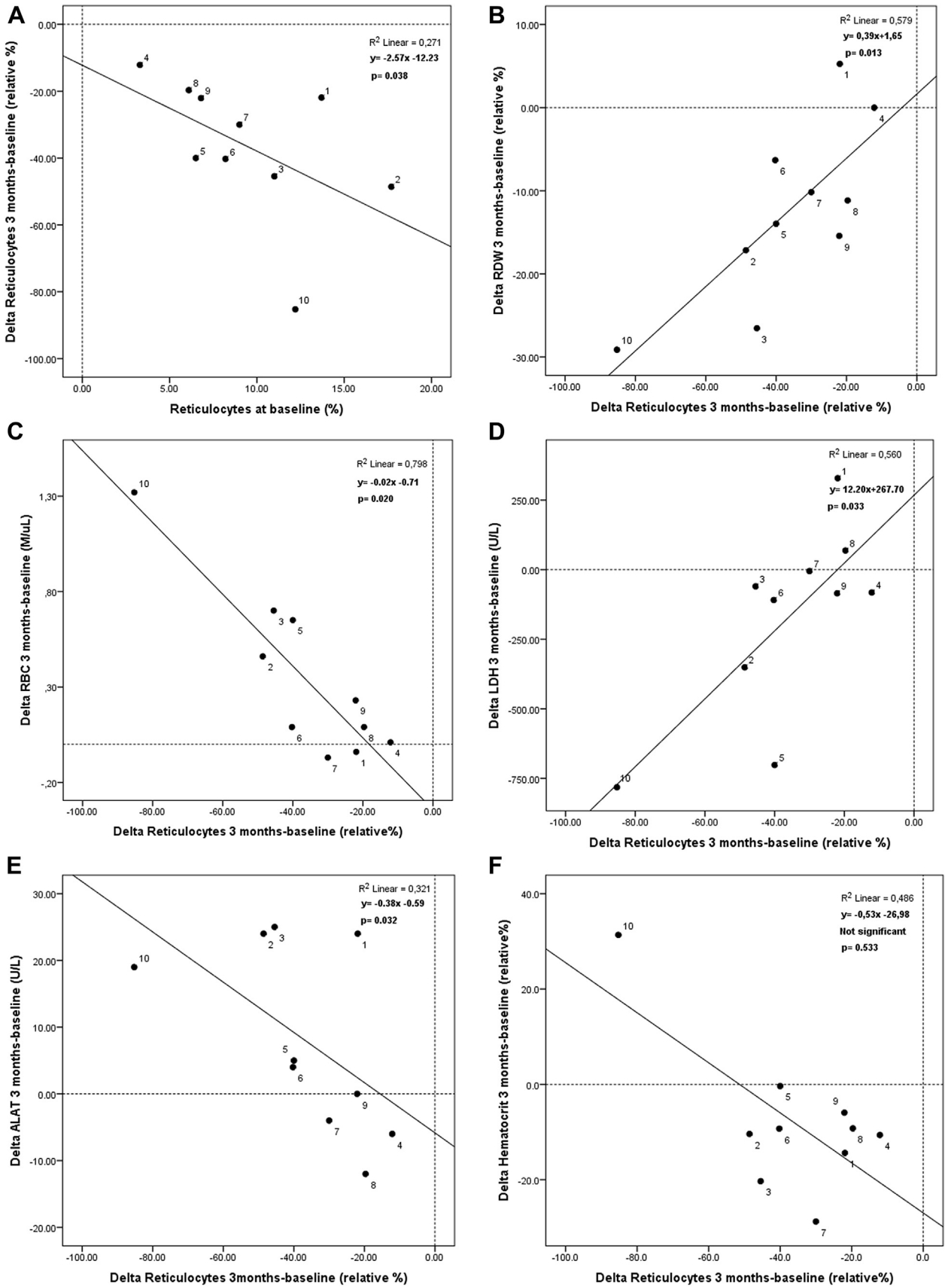

Fig. 2. Correlations of reticulocytes at baseline or percentage changes in reticulocytes from baseline to 3 months with changes in hematological parameters in the same period. Panel A: Significant negative correlation of reticulocytes at baseline (in %) with the relative change (in %) of reticulocytes from baseline to 3 months. The correlation suggests that patients with the highest reticulocyte percentages at baseline may have benefited most from astaxanthin supplementation. Panel B: Significant positive correlation between the

3.4. Correlations between changes in reticulocytes and hematological parameters

The correlation between reticulocytes at baseline and the percentage change in reticulocytes from baseline to 3 months is shown in Fig. 2, panel A ($p = 0.038$). The correlation indicates that the higher the baseline reticulocyte count, the higher percentage decrease in reticulocytes during astaxanthin supplementation. Reticulocyte count at baseline explained 27% of the reduction in reticulocytes from baseline to 3 months.

Positive correlations were found between the relative change in reticulocytes and the relative change in RDW ($p = 0.013$) (Fig. 2B) and LDH ($p = 0.033$) (Fig. 2, panel D), indicating that the decreases in reticulocytes from baseline to 3 months corresponded with decreases in both RDW and LDH. Significant negative correlations were found between the relative change in reticulocytes from baseline to 3 months and the relative changes in RBC ($p = 0.020$) (Fig. 2, panel C) and ALAT ($p = 0.032$) (Fig. 2, panel E) in the same period, indicating that the decrease in reticulocytes corresponded with increases of both RBC and ALAT. The relative change in reticulocytes from baseline to 3 months explained 58, 80, 56 and 32% of the changes in RDW, RBC, LDH and ALAT, respectively. The correlation between the relative change in reticulocytes from baseline to 3 months and the relative change in the hematocrit during the same period did not reach significance ($p = 0.533$) (Fig. 2, panel F).

4. Discussion

In this open label pilot study we supplemented 10 SCD patients in Dutch Sint Maarten with a daily oral dose of 8–12 mg astaxanthin during 3 months. The primary goal was to investigate whether astaxanthin incorporated into the RBC of these patients, and, secondary, whether this incorporation ameliorated the hemolytic component of the disease. Most importantly, we found that both plasma- and RBC-astaxanthin increased from baseline to 1–3 months (Table 1). Reticulocytes and RDW decreased from both baseline and 2 months–3 months, MCV from 2 to 3 months and MCH from baseline to 2 months. There were, however, no changes in plasma ADMA concentrations. To our knowledge, this was the first time that astaxanthin supplementation was studied in SCD patients.

4.1. Astaxanthin kinetics and incorporation into RBC

We found that both plasma- and RBC-astaxanthin had increased after 1 month of astaxanthin supplementation (Fig. 1, panels A and B), and decreased slightly thereafter until 3 months, although this decrease was not significant. The incorporation of astaxanthin in RBC confirms both the study of Nakagawa et al[37] and our previous study, where we showed that after a single oral 40 mg dose, astaxanthin increases rapidly in the plasma of healthy subjects (peak value at 8 h) and somewhat later in their RBC (peak at 12 h). Subsequently, astaxanthin decreased rapidly in both plasma and RBC, with estimated half-lives of 18 and 28 h, respectively, suggesting that to reach RBC steady state levels, daily supplementation may be needed, at least until a whole body equilibrium is reached, if any.[51] Consistent with our previous study, we found that both plasma and RBC levels were subject to high intra- and inter-individual variations (Fig. 1, panels A and B), which was attributed to non-standardized time difference between the last astaxanthin intake and blood sampling, fluctuating background astaxanthin intakes from the diet, variable bioavailability, large distribution volume, (induced) degradation, and possibly other factors.[51]

In our previous study in healthy subjects with a single dose of 40 mg astaxanthin and a subsequent 8 mg maintenance dose during 17 days,[51] we found that RBC contained medians of 44 and 49% from the plasma astaxanthin concentration, respectively. Also these data were subject to high intra- and inter-individual variations. These medians were, however, comparable with the estimated 43% calculated from the study of Nakagawa et al,[37] who supplemented healthy adults for 12 weeks with either 6 or 12 mg astaxanthin/day. In the present study with SCD patients, we found that the mean RBC concentration was 36% of the plasma concentration at baseline, but rose to almost double after 1 month supplementation ($p = 0.001$) (Table 1). Whether the seemingly deviant baseline distribution of astaxanthin between plasma and RBC and the observed response of this distribution relates to the time of sampling after daily oral supplementation, the higher RBC turnover in SCD or other factors, should be studied in a larger population.

4.2. Effects of astaxanthin on the hemolytic component

SCD is characterized by chronic hemolysis, high reticulocyte counts[52] and low RBC half-lives (5–10 days; reference: 25–40 days).[2] The observed modest decreases of reticulocytes and RDW from both baseline and 2 months–3 months, the MCH from baseline to 3 months and the MCV from 2 to 3 months, suggests that RBC turnover diminished slightly, but notably in the last month of supplementation. A more detailed evaluation suggested that patients with the highest percentage reticulocytes at baseline might have benefited most from astaxanthin supplementation, as they presented the highest relative decrease in reticulocytes (Fig. 2, panel A). This observation was consistent with the correlations found between the relative change in reticulocytes between 3 months and baseline and the changes across the same period in RDW (positive), RBC count (negative) and LDH (positive) (Fig. 2, panels B, C and D, respectively), altogether suggesting that there was a slight decrease in RBC turnover notably after 2 months of supplementation.

4.3. Possible adverse effects

Somewhat unexpectedly, we found that the change in reticulocytes across the 3 months supplementation period correlated with an increase in ALAT, suggesting that patients with the highest decreases in reticulocytes exhibited the highest ALAT increases (Fig. 2, panel E). Meanwhile, the group ALAT activity did not change, while all subjects presented ALAT values within the reference range (Table 1). We have at present no explanation for this suggested slight deterioration of liver function that came along with the decrease of the reticulocyte counts. No changes of ALAT activities

relative change of reticulocytes from baseline to 3 months (in %) and the change of the relative RDW (in %) in the same period. Panel C: Significant negative correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the change of RBC counts (in $10^6/\mu L$) in the same period. Panel D: Significant positive correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the change of LDH (in U/L) in the same period. Panel E: Significant negative correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the change of ALAT (in U/L) in the same period. The correlation suggests that patients with the greatest relative decreases in reticulocyte percentages presented the highest increases in ALAT. Panel F: Insignificant negative correlation between the relative change of reticulocytes from baseline to 3 months (in %) and the relative change of the hematocrit (in %) in the same period. The insignificance of this relation suggests that the relative decrease of reticulocyte percentage was not accompanied with an increase of the hematocrit, which is an important determinant of the rheology. Abbreviations: ALAT, alanine aminotransferase; LDH, lactate dehydrogenase; RBC, red blood cells; RDW, red blood cell distribution width.

have been reported in other astaxanthin supplementation studies. For instance, no changes in any transaminase were noted during the 12-week study of Nakagawa et al[37] with either 6 or 12 mg astaxanthin/day.

Fortunately, the change of reticulocytes did not correlate significantly with a concomitant change of the hematocrit (Fig. 2, panel F). Optimal oxygen transport efficiency for SCD patients occurs at a relatively low hematocrit. Sickle cells (either reversibly or irreversibly sickled cells) are intrinsically more rigid and viscous[53] and any augmentation of the hematocrit might be considered as an undesired effect.

We also found a decrease in arginine levels between 2 and 3 months of astaxanthin supplementation, though the levels at 3 months were not different from baseline. These changes did not cause any change in the ADMA/arginine ratio. Arginine is the substrate for NO synthesis, while ADMA inhibits NO formation. Consequently, any increase in the ADMA/arginine ratio, might reduce NO availability, causing a less favorable condition with less vasodilatation.[12]

4.4. Limitations

Apart from the astaxanthin kinetics, none of the presently reported changes in hematological and clinical chemical parameters, either positive or negative, might be related to the supplementation, since the current study was not placebo controlled. It is for instance known that these parameters may vary with season, with e.g. variable exposure to infectious agents.

5. Conclusions

This open label pilot study showed that oral astaxanthin supplementation increases astaxanthin concentrations in both plasma and RBC of SCD patients without any observed adverse reactions. Most promising, we found a slight reduction of the reticulocyte count after 3 months, probably indicating a lower hemolysis; while many of the patients reported that they 'felt better'. Whether these effects are causally related to the intervention is worth being investigated in a larger randomized controlled intervention study, where astaxanthin would be provided at a similar or higher dose during a trial that preferably lasts for more than 3 months. It might be even better to include astaxanthin into a supplemental mix with other antioxidants (e.g. low dose vitamin E,[54] beta-carotene, vitamin C and folic acid), minerals (selenium if necessary; and notably zinc[55,56];), amino acids (notably arginine[57,58]), fish oil[54,59] and vitamin D.[60] Antioxidants do not work on their own but are rather part of a yet poorly understood antioxidant network of free radical scavengers, quenchers and antioxidant enzymes and therefore, it seems improbable to find a single "magic bullet" to prevent or treat any disease associated with oxidative stress.[20]

Conflicts of interest

All authors have none to declare.

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References

1. Schnog JJ, Lard LR, Rojer RA, Van der Dijs FP, Muskiet FA, Duits AJ. New concepts in assessing sickle cell disease severity. *Am J Hematol*. 1998;58:61–66.
2. Serjeant GR. *Sickle Cell Disease*. 2nd ed. Oxford, UK: Oxford University Press; 1992.
3. Morris CR. Mechanisms of Vasculopathy in sickle cell disease and thalassemia. *Hematology*. 2008 January 1;2008:177–185.
4. Repka T, Hebbel RP. Hydroxyl radic formation by sickle erythrocyte membranes: role pathologic iron deposits cytoplasmic reducing agents. *Blood*. 1991 Nov 15;78:2753–2758.
5. Hebbel RP, Boogaerts MA, Eaton JW, Steinberg MH. Erythrocyte adherence to endothelium in sickle-cell anemia. A possible determinant of disease severity. *N Engl J Med*. 1980;302:992–995.
6. Turhan A, Weiss LA, Mohandas N, Coller BS, Frenette PS. Primary role for adherent leukocytes in sickle cell vascular occlusion: a new paradigm. *Proc Natl Acad Sci*. 2002 March 05;99:3047–3051.
7. Zennadi R. Role and regulation of sickle red cell interactions with other cells: ICAM-4 and other adhesion receptors. *Transfus Clinique Biologique*. 2008;15:23–28.
8. Aslan M, Ryan TM, Adler B, et al. Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. *Proc Natl Acad Sci U S A*. 2001;98:15215–15220.
9. Reiter C, Gladwin M. An emerging role for nitric oxide in sickle cell disease vascular homeostasis and therapy. *Curr Opin Hematol*. 2003;10:99–107.
10. Mori M, Gotoh T. Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem Biophys Res Commun*. 2000;275:715–719.
11. Morris S. Regulation of enzymes of the urea cycle and arginine metabolism. *Annu Rev Nutr*. 2002;22:87–105.
12. Morris CR, Kuypers FA, Larkin S, Vichinsky EP, Styles LA. Patterns of arginine and nitric oxide in patients with sickle cell disease with vaso-occlusive crisis and acute chest syndrome. *J Pediatr Hematology/oncology*. 2000;22:515–520.
13. Schnog JB, Teerlink T, van der Dijs FPL, Duits AJ, Muskiet FAJ. Plasma levels of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, are elevated in sickle cell disease. *Ann Hematol*. 2005;84:282–286.
14. Kato G, Wang Z, Machado R, Blackwelder W, Taylor J, Hazen S. Endogenous nitric oxide synthase inhibitors in sickle cell disease: abnormal levels and correlations with pulmonary hypertension, desaturation, haemolysis, organ dysfunction and death. *Br J Haematol*. 2009;145:506–513.
15. Landburg PP, Teerlink T, Biemond BJ, et al. Plasma asymmetric dimethylarginine concentrations in sickle cell disease are related to the hemolytic phenotype. *Blood Cells Mol Dis*. 2010;44:229–232.
16. D'Alecy L, Billecke S. Massive quantities of asymmetric dimethylarginine (ADMA) are incorporated in red blood cell proteins and may be released by proteolysis following hemolytic stress. *Blood Cells Mol Dis*. 2010;45:40.
17. Davids M, van Hell A, Visser M, Nijveldt R, van Leeuwen PAM, Teerlink T. Role of the human erythrocyte in generation and storage of asymmetric dimethylarginine. *Am J Physiol Heart Circ Physiol*. 2012;302:H1762–H1770.
18. Fibach E, Rachmilewitz E. The role of oxidative stress in hemolytic anemia. *Curr Mol Med*. 2008 Nov;8:609–619.
19. Egger G, Dixon J. Non-nutrient causes of low-grade, systemic inflammation: support for a 'canary in the mineshaft' view of obesity in chronic disease. *Obes Rev*. 2011;12:339–345.
20. Ruiz-Núñez B, Pruimboom L, Dijck-Brouwer DAJ, Muskiet FAJ. Lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low grade inflammation in an evolutionary context. *J Nutr Biochem*. 2013;24:1183–1201.
21. Hundekar P, Suryakar A, Karnik A, Katkam R, Joshi N, Ghone R. Level of nitric oxide and antioxidant vitamins in sickle cell anaemia patients. *Indian J Physiol Pharmacol*. 2012;56:125–129.
22. Ren H, Ghebremeskel K, Okpala I, Lee A, Ibegbulam O, Crawford M. Patients with sickle cell disease have reduced blood antioxidant protection. *Int J Vitam Nutr Res*. 2008 May;78:139–147.
23. Natta CL, Machlin LJ, Brin M. A decrease in irreversibly sickled erythrocytes in sickle cell anemia patients given vitamin E. *Am J Clin Nutr*. 1980;33:968–971.
24. Kalpravidh R, Siritanaratkul N, Insain P, et al. Improvement in oxidative stress and antioxidant parameters in beta-thalassemia/Hb E patients treated with curcuminoids. *Clin Biochem*. 2010;43:424–429.
25. Ohnishi ST, Ohnishi T. Vitro effects of aged garlic extract and other nutritional supplements on sickle erythrocytes. *The Journal of Nutrition*. 2001 March 01;131:1085S–1092S.
26. Nur E, Brandjes D, Teerlink T, et al. N-acetylcysteine reduces oxidative stress in sickle cell patients. *Ann Hematol*. 2012;91:1097–1105.
27. Bao B, Prasad AS, Beck FWJ, et al. Zinc supplementation decreases oxidative stress, incidence of infection, and generation of inflammatory cytokines in sickle cell disease patients. *Translat Res*. 2008;152:67–80.
28. Martin HD, Ruck C, Schmidt M, et al. Chemistry of carotenoid oxidation and free radical reactions. *Pure Appl Chem*. 1999;71:2253–2262.
29. Fassett R, Coombes J. Astaxanthin, oxidative stress, inflammation and cardiovascular disease. *Cardiology*. 2009;5:333–342.
30. McNulty H, Jacob RF, Mason RP. Biologic activity of carotenoids related to distinct membrane physicochemical interactions. *Am J Cardiol*. 2008;101(suppl 1):S20–S29.

31. Spiller GA, Dewell A. Safety of an astaxanthin-rich haematococcus pluvialis algal extract: a randomized clinical trial. *J Med Food*. 2003;6:51–56.
32. Guedes AC, Amaro H, Malcata FX. Microalgae as sources of high added-value compounds—a brief review of recent work. *Biotechnol Prog*. 2011;27:597–613.
33. Fassett R, Coombes J. Astaxanthin: a potential therapeutic agent in cardiovascular disease. *Marine Drugs*. 2011;9:447–465.
34. Fotouhi N, Meydani M, Santos MS, Meydani SN, Hennekens CH, Gaziano JM. Carotenoid and tocopherol concentrations in plasma, peripheral blood mononuclear cells, and red blood cells after long-term beta-carotene supplementation in men. *Am J Clin Nutr*. 1996;63:553–558.
35. Murata T, Tamai H, Morinobu T, et al. Determination of beta-carotene in plasma, blood cells and buccal mucosa by electrochemical detection. *Lipids*. 1992;27:840–843.
36. Nakagawa K, Kiko T, Hatade K, Sookwong P, Arai H, Miyazawa T. Antioxidant effect of lutein towards phospholipid hydroperoxidation in human erythrocytes. *Br J Nutr*. 2009;102:1280–1284.
37. Nakagawa K, Kiko T, Miyazawa T, et al. Antioxidant effect of astaxanthin on phospholipid peroxidation in human erythrocytes. *Br J Nutr*. 2011 Jan;31:1–9.
38. Park JS, Chyun JH, Kim YK, Line L, Chew B. Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. *Nutr Metab*. 2010;03-05:18.
39. Choi HD, Kim JH, Chang MJ, Kyu-Youn Y, Shin WG. Effects of astaxanthin on oxidative stress in overweight and obese adults. *Phytother Res*. 2011;25:1813–1818.
40. Guerra BA, Bolin AP, Otton R. Carbonyl stress and a combination of astaxanthin/vitamin C induce biochemical changes in human neutrophils. *Toxicology in Vitro*. 2012;26:1181–1190.
41. Kiko T, Nakagawa K, Satoh A, et al. Amyloid beta levels in human red blood cells. *PLoS ONE*. 2012;7:e49620.
42. *Haematococcus Astaxanthin: Health and Nutrition Applications. 1st Congress of the International Society for Applied Phycology/9th International Congress on Applied Phycology*. 2002.
43. Nir Y, Spiller G, Multz C. Effect of an astaxanthin containing product on rheumatoid arthritis. *J Am Coll Nutr*. 2002;21:490.
44. van Heyningen AM, Levenston MJ, Tamminga N, et al. Estimated incidence of sickle-cell disease in Aruba and St. Maarten suggests cost-effectiveness of a universal screening programme for St. Maarten. *West Indian Med J*. 2009 Sep;58:301–304.
45. Mercke Odeberg J, Lignell Å Pettersson A, Höglund P. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. *Eur J Pharm Sci*. 2003;7:299–304.
46. Nakagawa K, Kiko T, Hatade K, et al. Development of a high-performance liquid chromatography-based assay for carotenoids in human red blood cells: application to clinical studies. *Anal Biochem*. 2008 Oct 1;381:129–134.
47. Nakagawa K, Fujimoto K, Miyazawa T. beta-carotene as a high-potency antioxidant to prevent the formation of phospholipid hydroperoxides in red blood cells of mice. *Biochim Biophys Acta*. 1996;1299:110–116.
48. Teerlink T, Nijveldt R, de Jong S, van Leeuwen PAM. Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. *Anal Biochem*. 2002;303:131–137.
49. de Jong S, Teerlink T. Analysis of asymmetric dimethylarginine in plasma by HPLC using a monolithic column. *Anal Biochem*. 2006;353:287–289.
50. van der Zwan LP, Davids M, Scheffer P, Dekker J, Stehouwer CDA, Teerlink T. L-Homoarginine and L-arginine are antagonistically related to blood pressure in an elderly population: the Hoorn study. *J Hypertens*. 2013;31:1114–1123.
51. Ruiz-Núñez B, Schuitemaker GE, Dijck-Brouwer DAJ, Muskiet FAJ. Kinetics of plasma- and erythrocyte-astaxanthinin healthy subjects following a single and maintenance oral dose. *J Young Pharm*. 2013.
52. Bensinger TA, Gillette PN. Hemolysis in sickle cell disease. *Arch Intern Med*. 1974;133:624.
53. Verduzco L, Nathan D. Sickle cell disease and stroke. *Blood*. 2009;114:5117–5125.
54. Muskiet FA, Meiborg G, Schermer JG. Supplementation of patients with homozygous sickle cell disease with zinc, alpha-tocopherol, vitamin C, soybean oil, and fish oil. *Am J Clin Nutr*. 1991;54:736–744.
55. Zemel BS, Kawchak DA, Fung EB, Ohene-Frempong K, Stallings VA. Effect of zinc supplementation on growth and body composition in children with sickle cell disease. *Am J Clin Nutr*. 2002 February 01;75:300–307.
56. Brewer GJ, Brewer LF, Prasad AS. Suppression of irreversibly sickled erythrocytes by zinc therapy in sickle cell anemia. *J Lab Clin Med*. 1977;90:549–554.
57. Morris C, Kuypers F, Lavrisha L, et al. A randomized, placebo-control trial of arginine therapy for the treatment of children with sickle cell disease hospitalized with vaso-occlusive pain episodes. *Haematologica*. 2013;98:1375–1382.
58. Elias DB, Barbosa MC, Rocha LB, et al. L-arginine as an adjuvant drug in the treatment of sickle cell anaemia. *Br J Haematol*. 2013;160:410–412.
59. Daak A, Ghebremeskel K, Hassan Z, et al. Effect of omega-3 (n-3) fatty acid supplementation in patients with sickle cell anemia: randomized, double-blind, placebo-controlled trial. *Am J Clin Nutr*. 2013;97:37–44.
60. Osunkwo I, Ziegler T, Alvarez J, et al. High dose vitamin D therapy for chronic pain in children and adolescents with sickle cell disease: results of a randomized double blind pilot study. *Br J Haematol*. 2012;159:211–215.