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## Original Research

# Continuous astaxanthin intake reduces oxidative stress and reverses age-related morphological changes of residual skin surface components in middle-aged volunteers



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## ARTICLE INFO

## Article history:

Received 12 April 2017

Revised 4 October 2017

Accepted 5 October 2017

## Keywords:

Astaxanthin

Antioxidant

Antiaging effect

Skin surface

Corneocyte desquamation

Malondialdehyde

## ABSTRACT

Oxidative stress accelerates skin aging, and dietary supplementation with antioxidants may alleviate it. Morphological analysis of the residual skin surface components (RSSCs) allows detecting age-related changes in corneocyte desquamation, microbial presence, and lipid droplet size. We hypothesized that continuous ingestion of carotenoid antioxidant astaxanthin (4 mg/d) for 4 weeks could influence RSCC morphology and evaluated RSSC samples taken from middle-aged subjects before and after this dietary intervention. The study included 31 volunteers (17 men and 14 women) over the age of 40. RSSC samples were collected from the surface of the facial skin at the beginning (day 0) and end (day 29) of the study. In addition, blood samples were taken on days 0, 15, and 29 for measuring plasma levels of malondialdehyde that allowed assessing systemic oxidative stress. The results demonstrated that plasma malondialdehyde consistently decreased during astaxanthin consumption (by 11.2% on day 15 and by 21.7% on day 29). The analysis of RSSC samples has revealed significantly decreased levels of corneocyte desquamation ( $P = .0075$ ) and microbial presence ( $P = .0367$ ) at the end of the study. These phenomena as well as a significant ( $P = .0214$ ) increase in lipid droplet size were more strongly manifested among obese (body mass index  $>30 \text{ kg/m}^2$ ) subjects. All described RSSC changes correspond to a shift toward characteristics of skin associated with a younger age. The results confirm our hypothesis by demonstrating that continuous astaxanthin consumption produces a strong antioxidant effect resulting in facial skin rejuvenation which is especially pronounced in obese subjects.

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Abbreviations: BMI, body mass index; MDA, malondialdehyde; RSSC, residual skin surface component; SC, stratum corneum; SEM, standard error of mean.

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<https://doi.org/10.1016/j.nutres.2017.10.006>

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## 1. Introduction

Carotenoids constitute a large group of natural pigments that are present at high concentrations in plants, algae, and microorganisms [1-3]. It is now well established that these substances possess strong antioxidant and anti-inflammatory properties [1-3]. Carotenoids consumed with food were repeatedly proven to mitigate deteriorating effects of oxidative stress, nitrogen stress, and chronic inflammation, thus providing health-protecting and antiaging effects [1-6].

Astaxanthin (3,3'-dihydroxy-beta, beta-carotene-4,4'-dione) is a xanthophyll carotenoid produced by algae, bacteria, and fungi [7,8]. In the context of human nutrition, it is consumed with sea food, such as salmon, trout, crab, lobster, and shrimp [7,8], where it is predominantly present in the form of astaxanthin monoesters or diesters that may be hydrolyzed in the small intestine to facilitate absorption [9]. It is also evident that astaxanthin acts exclusively as a potent antioxidant, scavenging free radicals and other oxidants and, unlike some other carotenoids, never displaying pro-oxidant features [10,11]. Owing to these properties, it efficiently protects the lipid bilayer of cell membranes from peroxidation-caused damage [7,8,12]. In addition, astaxanthin exerts immunostimulatory action [13,14] and inhibits downstream inflammatory responses, thus producing beneficial anti-inflammatory effects [15,16]. Given the existence of abundant evidence supporting health-promoting properties of astaxanthin, it had been approved as a nutraceutical by the US Food and Drug Administration since 1999 [17]. The chlorophyte alga *Haematococcus pluvialis* accumulates the highest levels of astaxanthin in nature and currently serves the main natural source of this marine carotenoid used in the industrial production of health-promoting food supplements [17,18].

Human skin can be regarded as a major target of the beneficial antioxidant action of astaxanthin [19,20]. This is important because the development of new treatments allowing to prevent or even reverse skin aging has become an area of active exploration. Indeed, skin aging and associated cosmetic problems are currently recognized as a global challenge [21] directly related to rapidly increasing life expectancy all over the world [22].

The skin, especially its epidermal component, forms the protective barrier defending the human body from environmental factors, and the stratum corneum (SC), the outermost layer of the epidermis, is responsible for the key barrier functions [23]. Human SC consists of up to 20 layers of terminally differentiated corneocytes embedded in a lipid matrix [23], and it is remarkable that facial skin is characterized by a considerably thinner SC with only 7-11 layers of corneocytes [24]. In addition, a thin layer of a semiliquid substance known as the "acid mantle" (due to its mildly acidic pH) covers the surface of the SC. It is now established that this substance presents a mixture of lipids produced by the sebaceous glands and epidermal cells, desquamated corneocytes, and sweat; therefore, it was proposed to define it as "residual skin surface components" (RSSCs) [25].

Age-related changes of skin start to rapidly accelerate after the age of 30 [26] and involve gradual deterioration of epidermal barrier function [27,28] that progresses alongside

visible manifestations that comprise the loss of elasticity, dryness, wrinkling, atrophy, and laxity. It is believed that these phenomena are driven by 2 main mechanisms acting in combination: (a) intrinsic biological aging and (b) external exposure-caused photoaging, which is especially important for the face [21,29].

Dietary supplementation with carotenoids and, in particular, astaxanthin has repeatedly been demonstrated to produce antiaging effects by restoring skin moisture and elasticity, decreasing wrinkle formation, and improving epidermal barrier integrity [19,30-32]. However, elucidation of mechanisms behind these effects in human subjects is technically difficult because invasive methods of skin sampling are unsuitable for facial skin investigation. We addressed this problem by developing a simple technique for noninvasive RSSC sampling from the surface of the facial skin [33] and that could detect age-related changes in its morphological characteristics in both male [33] and female [34] subjects.

Presuming that continuous astaxanthin consumption results in reducing systemic oxidative stress, we hypothesized that this action could also alleviate aging-related changes on the surface of the SC, thus affecting RSSC composition. For testing this hypothesis, we aimed to comparatively evaluate RSSC morphology in middle-aged subjects before and after consumption of astaxanthin for 4 weeks. We also measured plasma levels of malondialdehyde (MDA), a recognized biomarker of systemic oxidative stress [35,36], for assessing systemic effects of astaxanthin supplementation.

## 2. Methods and materials

### 2.1. Study design

This collaborative study was undertaken by Lycotec Ltd (Cambridge, UK) and the Institute of Cardiology of the Ministry of Health of the Russian Federation (Saratov, Russian Federation). The enrollment of study participants was carried out in Saratov from the existing pool of healthy volunteers. Study protocol was approved by the local Ethics Committee (FGBU SarNIKI 18.02.2014), guaranteeing that the study conformed to the European Medicines Agency's *Guidelines for Good Clinical Practice*. All volunteers who agreed to participate in the study were informed about the purpose of the study and provided written informed consent.

The following inclusion criteria were applied to all individuals who volunteered to take part in the study: (1) only clinically healthy adult white men and women older than 40 years were considered for recruitment; (2) all study participants signed informed consent forms; (3) only non-smokers or light/moderate smokers ( $\leq 10$  cigarettes per day) were enrolled; and (4) all study participants were willing and able to comply with the protocol for the duration of the study.

The following exclusion criteria were applied: (1) unwillingness to sign informed consent forms; (2) inability to comply with the protocol for the duration of the study; (3) the presence of a significant medical condition (diagnosed cardiovascular or cerebrovascular disease, diabetes mellitus, oncological conditions, etc) or a disorder affecting skin (such

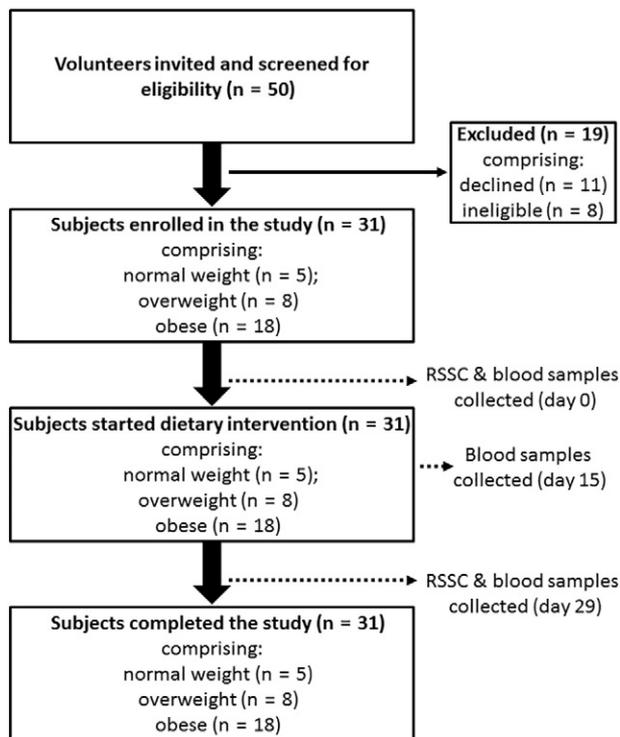


Fig. 1 – Flowchart showing study design.

as psoriasis, pronounced acne, and allergic skin conditions); (4) ongoing drug treatment (especially hormonal therapy); (5) excessive alcohol consumption (>35 UK units per week); (6) simultaneous participation in other studies involving dietary or pharmaceutical interventions; and (7) inability to tolerate phlebotomy.

Study design is presented in Fig. 1. From 50 individuals volunteering to take part in the study, 31 (17 male and 14 female subjects, 40–80 years of age) were recruited as eligible. All study participants initially had their body mass index (BMI) determined by measuring their body mass and height in the morning and then calculating the index in kilograms per square meter. Individuals with BMI less than 25 kg/m<sup>2</sup> were classified as subjects with normal weight, those with BMI in the range between 25 kg/m<sup>2</sup> and 30 kg/m<sup>2</sup> were classified as overweight, and BMI values greater than 30 kg/m<sup>2</sup> indicated obesity.

All study participants received 4-mg daily doses of astaxanthin (GA Astaxanthin capsules manufactured by Lycotec Ltd, Cambridge, UK) taken with the main evening meal for 4 weeks (from day 1 to day 28 of the study). This dose corresponds to the highest daily amount of astaxanthin approved by the European Food Safety Authority.

## 2.2. Sample collection and preparation

Blood samples were collected by phlebotomy from all study participants immediately before initiation of the dietary intervention (day 0) and after 2 weeks (day 15) and 4 weeks (day 29) of astaxanthin consumption. All blood collections were performed in the morning after night fast. The plasma was separated by centrifugation, aliquoted, and stored at –80°C until use.

For RSSC sample collection, all participants of the study were requested to avoid facial hygienic manipulations for 24 hours before material collection, which was carried out in the morning. These samples were collected only before initiation of astaxanthin supplementation (day 0) and at the end of the study (day 29). RSSC sample collection and preparation were performed according to previously described procedures [33,34]. Briefly, RSSC samples were collected using polyester swabs from the surface of the facial skin (the sides of the nose). During the procedure, 2 samples were taken (1 swab per side). Each collected sample was placed on the surface of a microscope slide. A second microscope slide was pressed against the surface of the first one to produce a pair of identical smears, which did not require fixation. All slides with collected samples (ie, 4 slides per study participant per time point) were coded to provide sample anonymity for blinded analysis. All collected samples were sent to the laboratory of Lycotec Ltd for further processing and eventual microscopic examination.

## 2.3. MDA determination in plasma samples

To assess the degree of oxidative stress in the plasma samples, thiobarbituric acid reactive substances assay kit (Cayman Chemical, MC, USA) was applied for MDA concentration determination. Assays were performed as previously reported [37], according to the instructions provided with the thiobarbituric acid reactive substances kit. MDA concentrations were assessed by colorimetry using Multiscan FC plate reader (Thermo Fisher Scientific Oy, Vantaa, Finland) as optical density at 532 nm. MDA standard provided with the kit was used for preparing standard dilution series for assay calibration.

The initial processing of MDA assay results included calibration curve generation and transformation of the absorbance values to MDA concentrations. These calculations were done using Skanit Software for Multiscan FC (AmsterChem, Amsterdam, Netherlands).

## 2.4. Morphological analysis of RSSC samples

For morphological analysis of RSSC samples, one slide of the first pair was stained with hematoxylin and eosin to identify any cells or cell remnants. The second slide was stained using Oil Red O (Lipid Stain, ab150678; Abcam, Cambridge, UK) for lipid visualization and lipid droplet size evaluation. One slide from the other pair was stained with crystal violet solution (Gram staining) to assess the level of microorganism presence. The remaining slides were kept unstained for possible future use.

All stained smears were examined microscopically by a highly experienced cytopathologist. Microscopy was performed using Olympus BX41 laboratory microscope (Olympus, Tokyo, Japan) at ×1000 magnification. The analysis included examining 40 fields of view within 1 smear. Dark field microscopy was applied for lipid crystal visualization. Photomicrographs were prepared using Olympus DP71 camera.

The analysis of typical structural elements of the RSSC comprised lipid droplet size measurement, counting characteristic lipid crystals and desquamated corneocytes, and evaluation of bacterial presence as previously described

**Table 1 – MDA concentration changes during continuous astaxanthin consumption in the whole study and subgroups of volunteers with different body composition (defined by BMI)**

Measured parameters	All subjects	BMI-defined subgroups		
		Normal weight	Overweight	Obese
No. of subjects (M/F)	31 (17/14)	5 (3/2)	8 (7/1)	18 (7/11)
Age (y)	61.45 ± 2.22	65.00 ± 4.76	53.75 ± 3.93	63.89 ± 3.05
BMI (kg/cm <sup>2</sup> )	30.39 ± 0.93	23.14 ± 0.67	28.41 ± 0.41	33.28 ± 1.04
MDA concentration (μmol/L) on:				
Day 0	0.304 <sup>a,b</sup> ± 0.014	0.256 <sup>d</sup> ± 0.021	0.318 <sup>e,f</sup> ± 0.025	0.312 <sup>h,i</sup> ± 0.021
Day 15	0.270 <sup>a,c</sup> ± 0.013	0.241 ± 0.020	0.282 <sup>e,g</sup> ± 0.024	0.272 <sup>h,j</sup> ± 0.019
Day 29	0.238 <sup>b,c</sup> ± 0.010	0.205 <sup>d</sup> ± 0.006	0.247 <sup>f,g</sup> ± 0.024	0.243 <sup>i,j</sup> ± 0.014

Values are expressed as means ± SEM.  
The different superscript letters (a-c; d; e-f; h-j) within columns indicate significant differences ( $P < .05$ ) in MDA concentrations when pairwise comparisons between time points were calculated using paired t test.

[33,34]. Briefly, lipid droplet size was assessed as the diameter of round droplets or long axis of oval droplets quantified using Cell<sup>^</sup>B imaging software (Digital Imaging Solutions, Olympus, Japan). Results of these measurements were summarized and expressed as average droplet size for each subject/time point. Likewise, counts of lipid crystals and desquamated corneocytes (averages per microscope field) were recorded for each subject/time point. The amount of bacteria per microscope field was assessed using our 5-grade Bacterial Presence Assessment Scale, expressing minimal amount of bacteria per microscope field as 1 and heavy bacterial presence as 5. This scale is described in detail in our previous article [33]. All samples used for microscopic examinations were anonymized to assure blind analysis.

The characteristics of the RSSC assessed per subject/time point thus comprised lipid droplet size, numbers of desquamated corneocytes and lipid crystals, and bacterial presence estimates (using Bacterial Presence Assessment Scale).

### 2.5. Statistical analyses

Data handling and statistical analyses of the results were carried out using IBM SPSS 19.0 statistical package (IBM Inc, Armonk, NY, USA). All quantitative results were calculated for the whole study population as well as for male and female subjects separately. Separate result assessments were also carried out for subgroups of subjects defined according to their BMI (normal weight, overweight, and obese).

Results of all quantitative measurements (or counting) were compared between the time points of the study. For MDA analysis, comparisons were made between days 0, 15, and 29. For RSSC assessment, only initial and final time points (ie, days 0 and 29) were compared. Descriptive statistics were used. Means, standard deviations, standard errors of the means (SEMs), medians, and range values as well as 95% confidence intervals were determined. Paired t test (2-sided  $P$  values calculated) was applied to determine statistical significance for the differences between time points. t test for independent means was used for comparing BMI-defined subgroups. Scatter diagrams were used for presenting individual results for all measurements in the RSSC assessment. Line chart was used for presenting MDA concentration dynamics during the study.

The minimum sample size for detecting quantitative changes of the RSSC components in a longitudinal (“before-after”) study was estimated at 27 based on a minimal difference of 15% between time points with a type I error  $\alpha = .5$  and power = 0.8 (type II error  $\beta = .2$ ) [38].

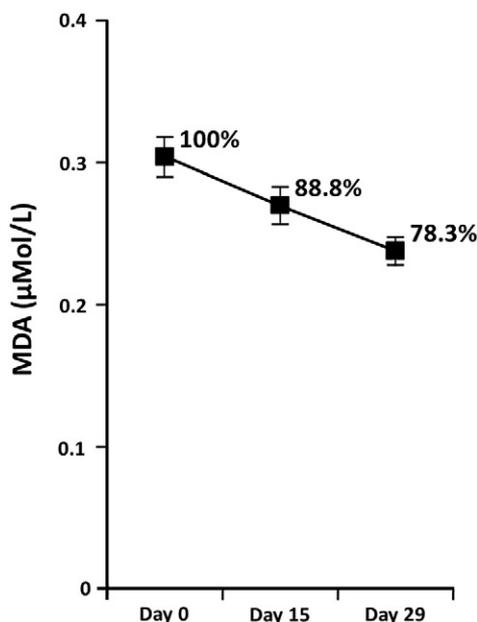
## 3. Results

### 3.1. General characteristics of study participants

The main criterion for volunteer recruitment in this study was age more than 40, when aging-related changes of the skin become clearly manifested, and the recruited volunteer group was relatively diverse in terms of age range (40-80), with the mean age 61.45 ± 2.22. It should also be noted that BMI determination indicated that the majority of study participants (18 subjects or 58%) could be classified as obese. Obesity was especially common among female volunteers (11 subjects or 79%). Table 1 demonstrates that only 5 study participants had normal weight, whereas 8 subjects were classified as overweight. The “overweight” subgroup had 7 male volunteers and only 1 female, and mean age in this subgroup was considerably lower (only 53.8 ± 3.93). The main purpose of the study was, however, unlikely to be affected by diversity within subgroups because dynamic changes of each parameter within each volunteer during astaxanthin consumption period did not appear to be either age or sex dependent.

### 3.2. Oxidative stress assessment by measuring MDA concentrations

Fig. 2 presents summary results on MDA concentration measurements at different time points of the study. It is evident that MDA levels steadily (almost linearly) decreased during astaxanthin consumption period, and comparisons between time points done using paired t test convincingly confirmed that MDA concentration values differed between days 0, 15, and 29 of the study with high significance (Table 1). When MDA levels on days 15 and 29 were expressed as percentage of the initial (day 0) measurement, the amount of the MDA detectable on day 15 was 88.8% of the initial, and this value further decreased to 78.3% at the end of the study (day 29).

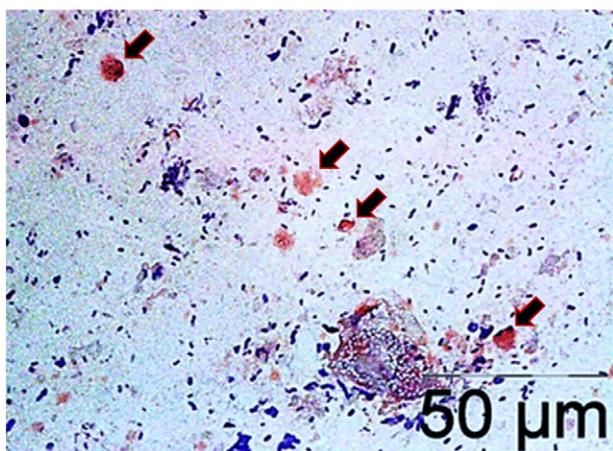


**Fig. 2 – MDA levels at 3 time points during the study (means and  $\pm$ SEM).**

It should be stressed that MDA concentration decrease was consistently observed regardless of body composition type (Table 1); however, absolute MDA levels tended to be lower in individuals with normal weight. Nevertheless, the difference between the subgroups did not reach statistical significance, which was not surprising given the small number of volunteers with normal weight.

### 3.3. RSSC analysis: lipid droplet size

Lipid droplets present in the collected RSSC samples could be easily identified by Oil Red O staining (Fig. 3) and measured during microscopy using Cell<sup>^</sup>B imaging software. Fig. 4A demonstrates results of lipid droplet size measurements



**Fig. 3 – Photomicrograph demonstrating the presence of lipid droplets in the collected RSSC material (lipid droplets are shown by arrows). Oil Red O staining.**

before astaxanthin intake initiation (day 0) and at the end of the study (day 29). Despite some variability of the results, it appeared that there was a relatively weak trend to increasing lipid droplet size at the end of the study. The difference between the 2 time points tested using paired t test, however, has not reached statistical significance ( $P = .0683$ ). The results also showed that there was no sex-dependent difference in lipid droplet size. Separate analysis of subgroups with different body composition revealed no statistically significant effect of astaxanthin in volunteers with normal weight or overweight individuals, whereas in the subgroup of obese subjects, a statistically significant ( $P = .0214$ ) increase of lipid droplet size was detected.

### 3.4. RSSC analysis: corneocyte desquamation

Evaluation of corneocyte desquamation in samples taken before astaxanthin consumption (day 0) and after 4 weeks of astaxanthin intake (day 29) has revealed a clear trend that is evident from Fig. 4B. Corneocyte desquamation levels at the end of the study were clearly reduced in most volunteers. This phenomenon was convincingly confirmed by paired t test, which has revealed a highly significant difference ( $P = .0075$ ). In some volunteers, the difference in desquamation levels was easy to see during microscopic analysis (Fig. 5A and B). When BMI-defined subgroups were assessed separately, the outcome of paired t test was statistically significant only for obese individuals ( $P = .0473$ ). No sex-related difference was revealed.

### 3.5. RSSC analysis: lipid crystals

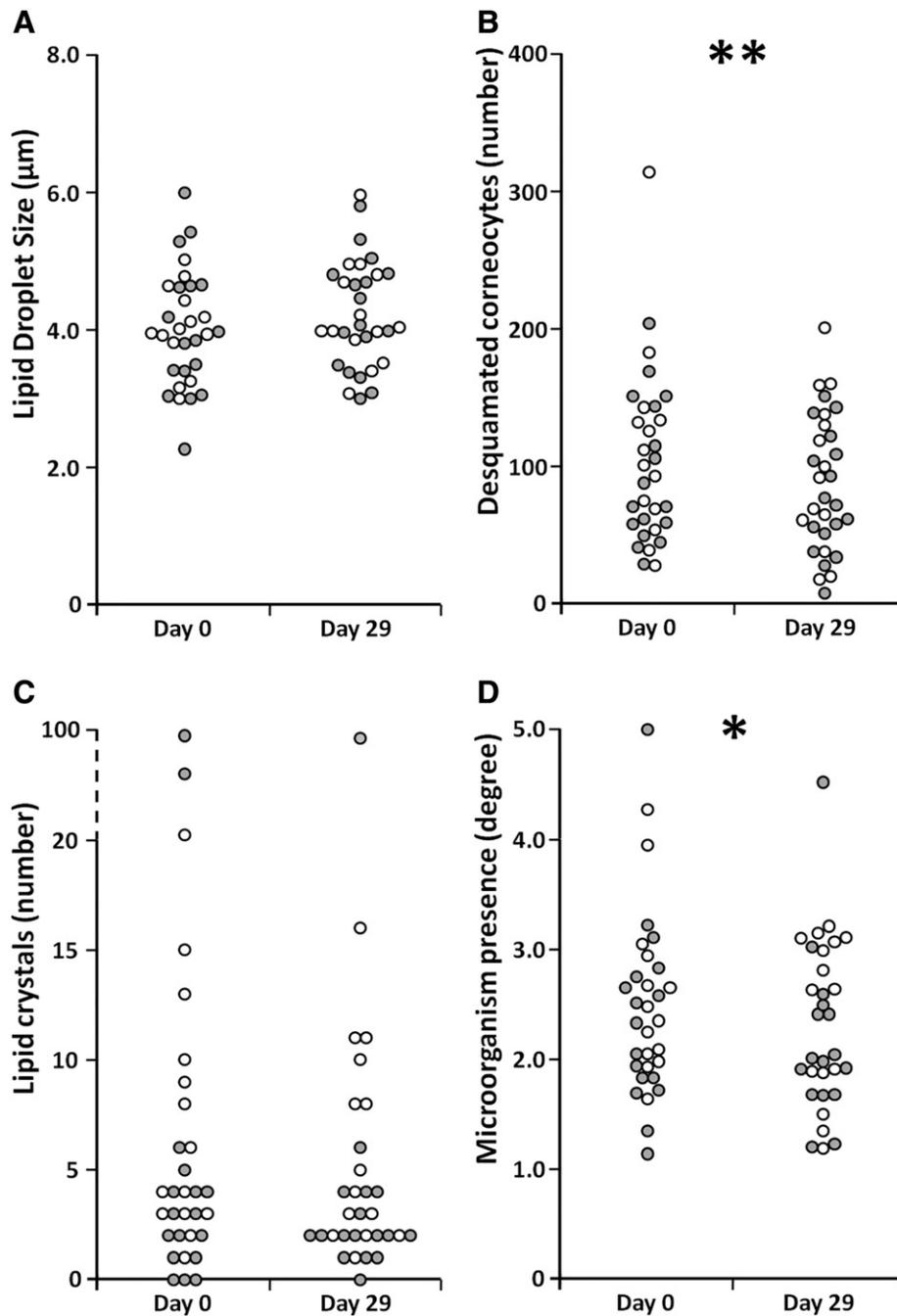
Although crystal structures could be identified in RSSC samples from the majority of volunteers, it was not possible to detect any clear trend associated with astaxanthin consumption. Distributions of lipid crystal numbers at the beginning and end of the study are presented in Fig. 4C. Neither BMI nor volunteer sex affected lipid crystal numbers.

### 3.6. RSSC analysis: microbial presence

The analysis of the microbial presence in RSSC samples was conducted using our previously described [33] semiquantitative scale. The obtained results are presented in Fig. 4D. We could find a statistically significant ( $P = .0367$ ) decrease of this parameter at the end of the study for the whole volunteer group. In addition, a statistically significant ( $P = .0312$ ) decrease in microbial presence was detected among obese volunteers. No sex-related differences could be found.

## 4. Discussion

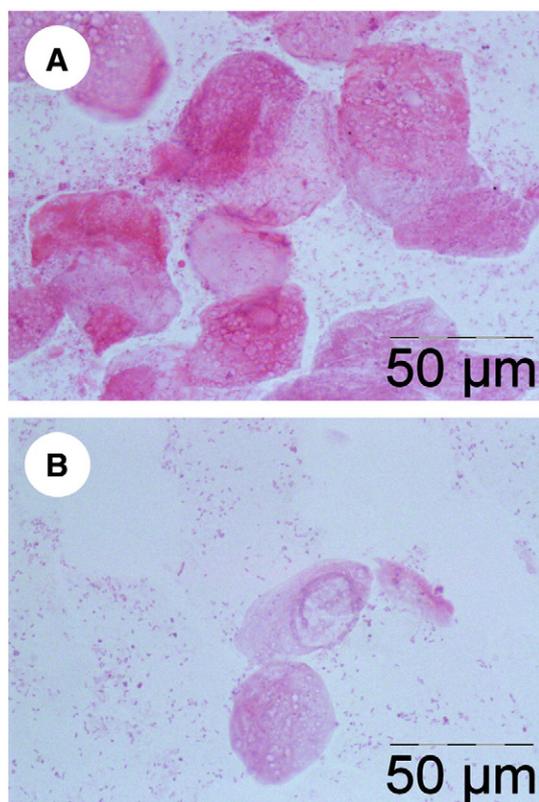
The use of dietary supplements for preventing skin aging and rendering skin youthful appearance is a fast-growing area of investigation that may lead to the development of new “nutricosmetic” products [39]. As astaxanthin is regarded as one of the most potent antioxidants exerting antiaging effects on skin [19,30–32], we were especially interested in detecting these effects at the level of RSSCs noninvasively collected



**Fig. 4 – Scatter diagrams showing individual result distribution (white symbols, female volunteers; gray symbols, male volunteers). A, Lipid droplet size as individual average values. B, Desquamated corneocyte counts as individual average counts per microscope field (\*\* $P = .0075$ ; comparison using paired t test.). C, Lipid crystal counts as individual average counts per microscope field. D, Microbial presence individual average estimates per microscope field according to our Bacterial Presence Assessment Scale [24] (\* $P = .0367$ ).**

from facial skin surface. Our previous work allowed concluding that RSSC samples taken from older individuals are characterized by more pronounced corneocyte desquamation, increased bacterial presence, and the accumulation of smaller lipid droplets [33,34]. In this study, we investigated effects of dietary supplementation with a moderate dose of astaxanthin that provides a stable elevation of its level in the plasma [40] (see also [41] that reviews interventions of this

type) and could demonstrate evident effects of this carotenoid on both general oxidative stress reduction and the RSSC characteristics. Indeed, astaxanthin consumption for 4 weeks resulted in a progressive significant decrease of MDA concentration in the plasma and appeared to inhibit corneocyte desquamation and decrease microbial presence by the end of the study. The latter RSSC-related phenomena can be interpreted as signs of a reversal of age-associated skin



**Fig. 5 – Photomicrographs of desquamated corneocytes observed in the same volunteer in the beginning of the study (A) and on day 29 (B). Hematoxylin and eosin staining.**

changes because enhanced corneocyte desquamation and more pronounced microbial presence in the RSSC were previously shown to characterize facial skin of older individuals [33,34].

The association of aging with elevated MDA levels is a well-proven fact repeatedly described by other authors [42–44]. Moreover, it is evident that dietary interventions involving antioxidants [45–47] and even positive lifestyle changes [48] can result in MDA decline. At the same time, relatively little was known so far about possible effects of astaxanthin on MDA concentration in the plasma of human subjects. Although astaxanthin was demonstrated to induce MDA decrease in several experimental studies [49–51], only one Korean group could demonstrate this effect in humans [52–54] before the present study.

Stratum corneum of the skin is one of many targets of the MDA, especially in the context of photo-oxidative stress [55], which certainly contributes to photoaging process and is clearly associated with hyperproliferation of keratinocytes followed by their enhanced desquamation [33,34,56]. It is assumed that oxidative protein damage increases protein degradation [29], thus facilitating desquamation of terminally differentiated corneocytes. This assumption is corroborated by observations of increased MDA levels in patients with psoriasis, a skin condition characterized by excessive epidermal proliferation and desquamation combined with dermal inflammation [57]. The outcome of our study indicates that continuous consumption of astaxanthin for only 4 weeks

results in a clear decrease of plasma MDA. It is apparent that this change reflects general antioxidant effect of astaxanthin. Oxidative damage in the skin is likely to decrease in parallel, which may entail declining keratinocyte desquamation. Molecular mechanisms of the latter phenomenon are not entirely clear; however, it is experimentally proven that astaxanthin is accumulated in the skin [58,59]. Komatsu et al [59] have recently shown that astaxanthin suppresses protease inhibitor expression in the SC of mice, thus stimulating proteolysis of filaggrin leading to the production of natural moisturizing factors that prevent corneocyte desquamation (see [60] for review on molecular aspects of desquamation). Further investigations are needed for proving the existence of this mechanism in humans.

It was shown in our previous study [33] that the presence of bacteria on the surface of the facial skin of older subjects was higher compared with the young ones. This most probably corresponds to age-related impairment of the immune system [61] that leads to mild inflammatory changes in the skin, thus creating favorable conditions for microbial growth. Decreasing microbial presence following astaxanthin consumption is consistent with the immunostimulatory [13,14] and anti-inflammatory [15,16] effects of this carotenoid.

We must admit that this study had limitations. There was no separate control (placebo) group, but by using the longitudinal (“before-after”) design, we assured that the initial (presupplementation) point of the study served as an internal control for postintervention measurements done in each study participant. In addition, a relatively small size of our study prevented us from appropriately comparing BMI-defined subgroups of subjects. Nevertheless, the results suggest that astaxanthin effects appeared to be more pronounced among individuals classified as obese. In this largest subgroup, we could observe not only significantly decreasing levels of MDA, corneocyte desquamation, and microbial presence but also a significant increase in the size of lipid droplets present in the RSSC, which also corresponds to previously identified characteristics of younger skin [33,34]. The latter observation may not be easy to interpret, but it is known that the amount of lipids on the surface of the epidermis declines with age [62,63], and the presence of lipophilic antioxidants normally found in sebum declines in parallel [62]. The trend to the enlargement of lipid droplets observed in obese volunteers in our study may reflect the antioxidant activity of the carotenoid that protects lipids in the sebum from peroxidation. It can be assumed that peroxidation-caused lipid damage and cross-linking result in an increased sebum viscosity. Therefore, the observed enlargement of lipid droplets is likely to be associated with viscosity reduction and could be interpreted as a sign of astaxanthin-induced reversal of age-related changes in the skin.

Although we need to be very cautious with conclusions because of the small size of “normal” and “overweight” subgroups in our study, it should be noted that correlations between higher BMI and increased oxidative stress and MDA were earlier detected by other investigators in postmenopausal women [64,65]. It appears to be logical to assume that obese individuals with high levels of oxidative stress may demonstrate a stronger response to the antioxidant effect of astaxanthin; however, making a firm conclusion on a stronger

skin-rejuvenating effect of astaxanthin in obese individuals may not be safe without performing larger confirmatory studies.

In our previous investigations of the RSSCs, we observed age-dependent changes in the presence of lipid crystals [33,34], but no significant effect of astaxanthin on these structures, which are most probably related to the sebum [66], could be detected in this study.

It can be concluded that by demonstrating that continuous consumption of astaxanthin for only 4 weeks resulted in RSSC changes consistent with the reversal of aging process, we have successfully confirmed our hypothesis, which can thus be accepted. The results also demonstrate that morphological investigation of the RSSCs is a useful tool for assessing skin-targeting effects of dietary factors. Although we strongly believe that these findings are very useful for developing new approaches to skin aging prevention, larger studies are needed for further characterizing the described phenomena.

## Acknowledgment

Volunteers who took part in the study are thanked for their cooperation. Alexandre Loktionov (DiagNodus Ltd, Cambridge, UK) is thanked for his critical advice and help in manuscript preparation. This research was supported by Lycotec Ltd. The authors of this paper are associated with either Lycotec Ltd (NEC, VAK, NHK, IMP) or DiagNodus Ltd (TYB), which are private companies. Therefore, all authors may have a potential financial conflict of interest.

## REFERENCES

- [1] McNulty H, Jacob RF, Mason RP. Biologic activity of carotenoids related to distinct membrane physicochemical interactions. *Am J Cardiol* 2008;101:20D–9D [suppl].
- [2] Rao AV, Rao LG. Carotenoids and human health. *Pharmacol Res* 2007;55:207–16.
- [3] Kaulmann A, Bohn T. Carotenoids, inflammation, and oxidative stress—implications of cellular signalling pathways and relation to chronic disease prevention. *Nutr Res* 2014;34:907–29.
- [4] Woodside JV, McGrath AJ, Lyner N, McKinley MC. Carotenoids and health in older people. *Maturitas* 2015;80:63–8.
- [5] Wolak T, Paran E. Can carotenoids attenuate vascular aging? *Vascul Pharmacol* 2013;59:63–6.
- [6] Raposo MF, de Moraes AM, de Moraes RM. Carotenoids from marine microalgae: a valuable natural source for the prevention of chronic diseases. *Mar Drugs* 2015;13:5128–55.
- [7] Kidd P. Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging potential. *Altern Med Rev* 2011;16:355–64.
- [8] Yuan J-P, Peng J, Yin K, Wang J-H. Potential health-promoting effects of astaxanthin: a high value carotenoid mostly from microalgae. *Mol Nutr Food Res* 2011;55:150–65.
- [9] Sugawara T, Yamashita K, Asai A, Nagao A, Shiraiishi T, Imai I, et al. Esterification of xanthophylls by human intestinal Caco-2 cells. *Arch Biochem Biophys* 2009;483:205–12.
- [10] Martin HD, Ruck C, Schmidt M, Sell S, Beutner S, Mayer B, et al. Chemistry of carotenoid oxidation and free radical reactions. *Pure Appl Chem* 1999;71:2253–62.
- [11] Beutner S, Bloedorn B, Frixel S, Hernandez Blanco I, Hoffman T, Martin HD, et al. Quantitative assessment of antioxidant properties of natural colorants and phytochemicals: carotenoids, flavonoids, phenols and indigoids. The role of  $\beta$ -carotene in antioxidant functions. *J Sci Food Agric* 2001;81:559–68.
- [12] Gammone MA, Riccioni G, D’Orazio N. Marine carotenoids against oxidative stress: effects on human health. *Mar Drugs* 2015;13:6226–46.
- [13] Speranza L, Pesce M, Patruno A, Franceschelli S, de Lutiis MA, Grilli A, et al. Astaxanthin treatment reduced oxidative induced pro-inflammatory cytokines secretion in U937: SHP-1 as a novel biological target. *Mar Drugs* 2012;10:890–9.
- [14] Lin KH, Lin KC, Lu WJ, Thomas PA, Jayakumar T, Sheu JR. Astaxanthin, a carotenoid, stimulates immune responses by enhancing IFN- $\gamma$  and IL-2 secretion in primary cultured lymphocytes in vitro and ex vivo. *Int J Mol Sci* 2015;17. <https://doi.org/10.3390/ijms17010044> [pii:E44].
- [15] Yasui Y, Hosokawa M, Mikami N, Miyashita K, Tanaka T. Dietary astaxanthin inhibits colitis and colitis-associated colon carcinogenesis in mice via modulation of the inflammatory cytokines. *Chem Biol Interact* 2011;193:79–87.
- [16] Miyachi M, Matsuno T, Asano K, Mataga I. Anti-inflammatory effects of astaxanthin in the human gingival keratinocyte line NDUSD-1. *J Clin Biochem Nutr* 2015;56:171–8.
- [17] Lorenz RT, Cysewski GR. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol* 2000;18:160–7.
- [18] Guerin M, Huntley ME, Olaizola M. *Haematococcus* astaxanthin: applications for human health and nutrition. *Trends Biotechnol* 2003;21:210–6.
- [19] Tominaga K, Hongo N, Karato M, Yamashita E. Cosmetic benefits of astaxanthin on human subjects. *Acta Biochim Pol* 2012;59:43–7.
- [20] Tominaga K, Hongo N, Fujishita M, Takahashi Y, Adachi Y. Protective effects of astaxanthin on skin deterioration. *J Clin Biochem Nutr* 2017;61:33–9.
- [21] Al-Nuaimi Y, Sherratt MJ, Griffiths CEM. Skin health in older age. *Maturitas* 2014;79:256–64.
- [22] Christensen K, Doblhammer C, Rau R, Vaupel JW. Ageing populations: the challenges ahead. *Lancet* 2009;374:1196–208.
- [23] Menon GK, Cleary GW, Lane ME. The structure and function of the stratum corneum. *Int J Pharm* 2012;435:3–9.
- [24] Tagami H. Location-related differences in structure and function of the stratum corneum with special emphasis on those of the facial skin. *Int J Cosmet Sci* 2008;30:413–34.
- [25] Shetage SS, Traynor MJ, Brown MB, Raji M, Graham-Kalio D, Chilcott RP. Effect of ethnicity, gender and age on the amount and composition of residual skin surface components derived from sebum, sweat and epidermal lipids. *Skin Res Technol* 2014;20:97–107.
- [26] Kuwazuru O, Miyamoto K, Yoshikawa N, Imayama S. Skin wrinkling morphology changes suddenly in the early 30s. *Skin Res Technol* 2012;18:495–503.
- [27] Luebbberding S, Krueger N, Kerscher M. Age-related changes in skin barrier function – quantitative evaluation of 150 female subjects. *Int J Cosmet Sci* 2013;35:183–90.
- [28] Luebbberding S, Krueger N, Kerscher M. Age-related changes in male skin: quantitative evaluation of one hundred and fifty male subjects. *Skin Pharmacol Physiol* 2014;27:9–17.
- [29] Vierkötter A, Krutmann J. Environmental influences on skin aging and ethnic-specific manifestations. *Dermatoendocrinology* 2012;4:227–31.
- [30] Suganuma K, Nakajima H, Ohtsuki M, Imokawa G. Astaxanthin attenuates the UVA-induced up-regulation of matrix-metalloproteinase-1 and skin fibroblast elastase in human dermal fibroblasts. *J Dermatol Sci* 2010;58:136–42.
- [31] Yoon HS, Cho HH, Cho S, Lee SR, Shih MH, Chung JH. Supplementing with dietary astaxanthin combined with collagen hydrolysate improves facial elasticity and decreases matrix metalloproteinase-1 and -12 expression: a comparative study with placebo. *J Med Food* 2014;17:810–6.

- [32] Phetcharat L, Wongsuphasawat K, Winther K. The effectiveness of a standardized rose hip powder, containing seeds and shells of *Rosa canina*, on cell longevity, skin wrinkles, moisture, and elasticity. *Clin Interv Aging* 2015;10:1849–56.
- [33] Chalyk NE, Bandaletova TY, Kyle NH, Petyaev IM. Age-related differences in morphological characteristics of residual skin surface components collected from the surface of facial skin of healthy male volunteers. *Skin Res Technol* 2017;23:212–20.
- [34] Chalyk NE, Bandaletova TY, Kyle NH, Petyaev IM. Morphological characteristics of residual skin surface components collected from the surface of facial skin in women of different age. *Ann Dermatol* 2017;29:454–61.
- [35] Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjean P. Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. *Clin Chem* 1997;43:1209–14.
- [36] Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* 2005;15:316–28.
- [37] Petyaev IM, Dovgalevsky PY, Klochkov VA, Chalyk NE, Kyle N. Whey protein lysosome formulation improves vascular functions and plasma lipids with reduction of markers of inflammation and oxidative stress in prehypertension. *ScientificWorldJournal* 2012. <https://doi.org/10.1100/2012/269476>.
- [38] Rosner B. *Fundamentals of biostatistics*. 7th ed. Brooks/Cole Cengage Learning; 2011.
- [39] Draelos ZD. Nutrition and enhancing youthful-appearing skin. *Clin Dermatol* 2010;28:400–8.
- [40] 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;75:1856–8.
- [41] Kishimoto Y, Yoshida H, Kondo K. Potential anti-atherosclerotic properties of astaxanthin. *Mar Drugs* 2016;14:E35. <https://doi.org/10.3390/md14020035>.
- [42] Rodriguez-Martinez MA, Ruiz-Torres A. Homeostasis between lipid peroxidation and antioxidant enzyme activities in healthy human aging. *Mech Ageing Dev* 1992;66:213–22.
- [43] Inal ME, Kanbak G, Sunal E. Antioxidant enzyme activities and malondialdehyde levels related to aging. *Clin Chim Acta* 2001;305:75–80.
- [44] Traverso N, Patriarca S, Balbis E, Furfaro AL, Cottalasso D, Pronzato MA, et al. Anti malondialdehyde-adduct immunological response as a possible marker of successful aging. *Exp Gerontol* 2003;38:1129–35.
- [45] Wang MX, Jiao JH, Li ZY, Liu RR, Shi Q, Ma L. Lutein supplementation reduces plasma lipid peroxidation and C-reactive protein in healthy non-smokers. *Atherosclerosis* 2013;227:380–5.
- [46] Panahi Y, Hosseini MS, Khalili N, Naimi E, Majeed M, Sahebkar A. Antioxidant and anti-inflammatory effects of curcuminoid-piperine combination in subjects with metabolic syndrome: a randomized control trial and updated meta-analysis. *Clin Nutr* 2015;34:1101–8.
- [47] Matsuura T, Takayama K, Kaneko H, Ye F, Fukukita H, Tsunekawa T, et al. Nutritional supplementation inhibits the increase of malondialdehyde in patients with wet age-related macular degeneration. *Oxid Med Cell Longev* 2017;9548767. <https://doi.org/10.1155/2017/9548767>.
- [48] Bhatnagar A, Tripathi Y, Kumar A. Change in oxidative stress of normotensive elderly subjects following lifestyle modifications. *J Clin Diagn Res* 2016;10:CC09–13.
- [49] Sowmya PR, Arathi BP, Vijay K, Baskaran V, Lakshminarayana R. Role of different vehicles in carotenoids delivery and their influence on cell viability, cell cycle progression, and induction of apoptosis in HeLa cells. *Mol Cell Biochem* 2015;406:245–53.
- [50] Sila A, Ghlissi Z, Kamoun Z, Makni M, Nasri M, Bougateg A, et al. Astaxanthin from shrimp by-products ameliorates nephropathy in diabetic rats. *Eur J Nutr* 2015;54:301–7.
- [51] Xuan RR, Nu TT, Chen HM. Astaxanthin blocks preeclampsia progression by suppressing oxidative stress and inflammation. *Mol Med Rep* 2016;14:2697–704.
- [52] Kim JH, Chang MJ, Choi HD, Youn YK, Kim JT, Oh JM, et al. Protective effects of *Haematococcus astaxanthin* on oxidative stress in healthy smokers. *J Med Food* 2011;14:1469–75.
- [53] Choi HD, Youn YK, Shin WG. Positive effects of astaxanthin on lipid profiles and oxidative stress in overweight subjects. *Plant Foods Hum Nutr* 2011;66:363–9.
- [54] 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;25:1813–8.
- [55] Thiele JJ, Schroeter C, Hsieh SN, Podda M, Packer L. The antioxidant network of the stratum corneum. *Curr Probl Dermatol* 2001;29:26–42.
- [56] Chu M, Kollias N. Documentation of normal stratum corneum scaling in an average population: features of differences among age, ethnicity and body size. *Br J Dermatol* 2011;164:497–507.
- [57] Pujari VM, Ireddy S, Itagi I, Kumar HS. The serum levels of malondialdehyde, vitamin E and erythrocyte catalase activity in psoriasis patients. *J Clin Diagn Res* 2014;8:CC14–6. <https://doi.org/10.7860/JCDR/2014/10912.5085>.
- [58] Petri D, Lindebye AK. Tissue distribution of astaxanthin in rats following exposure to graded levels in the feed. *Comp Biochem Physiol C Toxicol Pharmacol* 2007;145:202–9.
- [59] Komatsu T, Sasaki S, Manabe Y, Hirata T, Sugawara T. Preventive effect of dietary astaxanthin on UVA-induced skin photoaging in hairless mice. *PLoS One* 2012;12:e0171178. <https://doi.org/10.1371/journal.pone.0171178>.
- [60] Kim BE, Leung DY. Epidermal barrier in atopic dermatitis. *Allergy Asthma Immunol Res* 2012;4:12–6. <https://doi.org/10.4168/aair.2012.4.1.12>.
- [61] Zapata HJ, Quagliarello VJ. The microbiota and microbiome in aging: potential implications in health and age-related diseases. *J Am Geriatr Soc* 2015;63:776–81.
- [62] Passi S, De Pità O, Puddu P, Littarru GP. Lipophilic antioxidants in human sebum and aging. *Free Radic Res* 2002;36:471–7.
- [63] Boireau-Adamezyk E, Baillet-Guffroy A, Stamatias GN. Age-dependent changes in stratum corneum barrier function. *Skin Res Technol* 2014;20:409–15.
- [64] Mittal PC, Kant R. Correlation of increased oxidative stress to body weight in disease-free post menopausal women. *Clin Biochem* 2009;42:1007–11.
- [65] Goy B, Atmaca M, Aslan M, Ucler R, Alay M, Seven I, et al. Relationship between serum DHEAS and oxidative stress levels of body mass index in healthy postmenopausal women. *Redox Rep* 2016;21:61–6.
- [66] González-Serva A, Kroumpouzou G. Demonstration of polarisable crystals in fresh comedonal extracts: sebum crystallizes. *Acta Derm Venereol* 2004;84:418–21.