

# Non-Invasive Immunofluorescence Assessment of Lycopene Supplementation Status in Skin Smears

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Circulating lycopene level is negatively associated with the prevalence of cardiovascular disease, cancers (prostate and breast), type 2 diabetes mellitus, and aging. Traditionally, lycopene is measured in biological specimens by a combination of high-performance liquid chromatography (HPLC) and mass spectrometry methods. Moreover, as we recently reported, tissue/cell lycopene depositions can be observed by the immunohistochemistry method with a newly developed monoclonal antibody (mAb) against lycopene. A main objective of this study is to evaluate the performance of a new noninvasive immunofluorescence (IF) lycopene quantification skin test with mAbs against lycopene versus HPLC lycopene assay of serum lycopene in volunteers subjected to lycopene supplementation and represents its novelty. For this purpose, 32 healthy volunteers, 30–40 years old, were supplemented with lycopene ( $n = 15$ ) or placebo ( $n = 17$ ) for a period of 4 weeks. It was found that lycopene supplementation leads to a significant increase in serum lycopene concentration after 2 and 4 weeks by 2.6- and 3.4-fold over control, respectively. This was accompanied by a concordant step-wise rise in IF staining of skin corneocytes and sebum, quantifiable by arbitrary IF scores. Placebo supplementation did not affect serum lycopene values or intensity of IF staining of the skin samples. There was 86.6% agreement in paired HPLC/IF variants for the intermediate time point and 80.0% agreement at the end of the study in the lycopene group. Intraclass correlation between paired values in this group was +0.49 for the 2-week time point and +0.63 for the end point. These results indicate that the new antibody-based skin assay can be used for rapid detection of lycopene deficiencies. Moreover, the noninvasive nature of the skin swab test would allow using it to monitor, optimize, and personalize lycopene supplementation protocol of risk groups in the general population.

**Keywords:** immunofluorescence, lycopene, skin smears, quantification

## Introduction

LYCOPENE IS A MEVALONATE-DERIVED tetraterpene carotenoid synthesized in certain plants (tomatoes, papaya, and watermelon) and bacteria by conjugation of eight isoprene units into a 40-carbon molecule.<sup>(1)</sup> Mammalian species cannot synthesize lycopene and acquire it exclusively from dietary sources.<sup>(1,2)</sup> Eleven linearly aligned hydrogen double bonds in the structure of the molecule confer an extraordinary antioxidant potential on lycopene whose antiradical properties exceed the antioxidant potential of other nonpolar carotenoids multifold.<sup>(3,4)</sup>

Regular lycopene consumption has been shown to have a number of health benefits. Supplementation may have a positive impact on the initiation, development, and outcomes of

cardiovascular disease (CVD), some forms of cancer, type 2 diabetes, and respiratory disorders.<sup>(5–9)</sup> These results are in agreement with epidemiological observations, suggesting that higher dietary intake of lycopene is associated with reduced prevalence of CVD, prostate, and breast cancers.<sup>(10–12)</sup> It is important to note that the circulating lycopene levels have a better prognostic value for health outcomes than the values of dietary lycopene intake.<sup>(4,11,12)</sup> Such a discrepancy originates from the low bioavailability rate of the lycopene molecule.<sup>(4,9)</sup> Lycopene is a highly hydrophobic and poorly absorbed nutrient localized in the barely digestible chromoplasts of plant cells. A significant proportion of ingested lycopene is oxidized and undergoes irreversible enzymatic degradation and/or oxidation by intestinal enzymes with further fecal disposal.<sup>(13,14)</sup>

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Thus, even high intake of lycopene-rich dietary constituents may not always be sufficient to prevent or overcome existing lycopene deficiency, especially in elderly persons and those who are overweight, obese, or have metabolic syndrome. The blood level of lycopene and its dietary intake have been shown to be low in the general population even in developed Western countries, including the United States.<sup>(4,15)</sup>

Therefore, widespread and regular assessment of blood lycopene levels and identification of individuals with lycopene deficiency in the general population become a challenging task in preventive medicine. Modern laboratory methods for lycopene measurement in serum or plasma specimens are based on high-performance liquid chromatography (HPLC) with further mass spectrometry, an expensive and labor-intensive procedure requiring highly trained personnel. Recently we developed a monoclonal antibody (mAb) against lycopene,<sup>(16)</sup> which allows for the observation of lycopene depositions in cultured cells and some tissues. Therefore, this mAb against lycopene can be potentially useful for the development of new lycopene quantification assays to verify lycopene supplementation status. Here we report that a lycopene-specific mAb, conjugated with a fluorescent label, can be used for direct immunofluorescence (IF) assessment of lycopene content in corneocytes and sebum obtained from the skin swabs. As we show hereunder, changes in serum lycopene concentration develop in close interrelation with changes in skin lycopene content. These results constitute a novelty in our research and indicate that this new noninvasive test can be potentially used for the identification of individuals with lycopene deficiency as well as for the monitoring, optimization, and personalization of lycopene supplementation protocol in the general population.

## Materials and Methods

### Study design

The study was designed as a double arm prospective placebo-controlled interventional pilot nutraceutical trial performed under a protocol approved by the Local Ethical Committee (FGBU SarNIK 15.06.2016) and registered (ISRCTN89815519). All study participants were informed of the study objectives and study products and signed written consent before enrollment. However, none of them was informed about group assignment or whether they received placebo or lycopene capsules. All volunteers underwent physical and laboratory examinations and were asked about their medical history and socioeconomic background.

**Subjects and inclusion/exclusion criteria.** Fifty-seven qualifying individuals were originally enrolled in the study (Fig. 1). Sixteen volunteers were excluded after initial screening due to noncompliance with the inclusion/exclusion criteria. Forty-one volunteers were randomized into two study groups using a simple computerized block randomization method. Nine individuals were not able to complete the study for various reasons. Finally, the results obtained from 17 individuals in the placebo group and 15 individuals in the lycopene supplementation group were analyzed.

Inclusion criteria were Caucasian males or females aged from 30 to 40 years with normal BMI; signed consent form; no history or current food allergies, antihypertensive, lipid-lowering, or antidiabetic drugs; and willingness and ability to

comply with the study protocol. Exclusion criteria were any dermatological disease or disorder, excessive consumption of tomato-containing food products (tomatoes, pizza, and tomato juice), 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, heavy tobacco use (smoking >10 cigarettes per day), and intolerance of tomatoes or dietary restrictions.

All volunteers were asked to abstain from consumption of tomato-containing products as well as tomatoes, papaya, watermelon, carrots, parsley, and asparagus for 14 days before beginning the study and during the study itself. After completion of the run-in period, patients were given the study products. In addition, after recruitment, all volunteers were advised to avoid use of any cosmetics and facial creams. Face washes with warm water and regular soap were allowed on a daily basis.

### Study groups

The total duration of the study was 4 weeks. Participants from the first group received a set of placebo capsules, whereas participants from the second group received a set of highly bioavailable lycopene capsules (7 mg, GA Lycopene; Lycotec Ltd., Cambridge, United Kingdom). All volunteers underwent medical evaluation and laboratory investigation at enrollment (prior to the run-in period), on day "0" of the trial (after the run-in period), and at 2 and 4 weeks after trial initiation. Compliance with the protocol was confirmed during participant visits at the intermediate and final time points of the study as well as by random phone call interviews during the study.

### Study products

Lycopene oleoresin all-trans isomers was purchased from LycoRed (LycoRed, Switzerland) and kept in oxygen-free containers at  $-80^{\circ}\text{C}$ . A highly bioavailable lycosome formulation of GA lycopene, prepared as described previously,<sup>(17)</sup> was administered orally, 7 mg daily, in the evening with dinner. Capsules containing an inert and irrelevant compound were used in the placebo group with the same mode of oral intake.

### Blood and facial smear collection

Blood was collected in the morning from arm veins of the participants after overnight fast. The serum was separated from the rest of the clotted mass by centrifugation and then coded aliquots were stored at  $-80^{\circ}\text{C}$  before blinded analysis.

All study participants were requested to avoid any facial hygienic manipulations for 24 hours before sampling. Skin surface sample collection was carried out in the morning alongside blood collection as previously described.<sup>(18)</sup> In brief, samples were collected using polyester swabs from the facial skin (the lateral surface of the nose). During the procedure, two samples were taken (one smear per side). Each sample collected was placed on the surface of a microscope slide. A second microscope slide was pressed against the surface of the first slide. This procedure provided a pair of identical smears. All slides with collected samples were

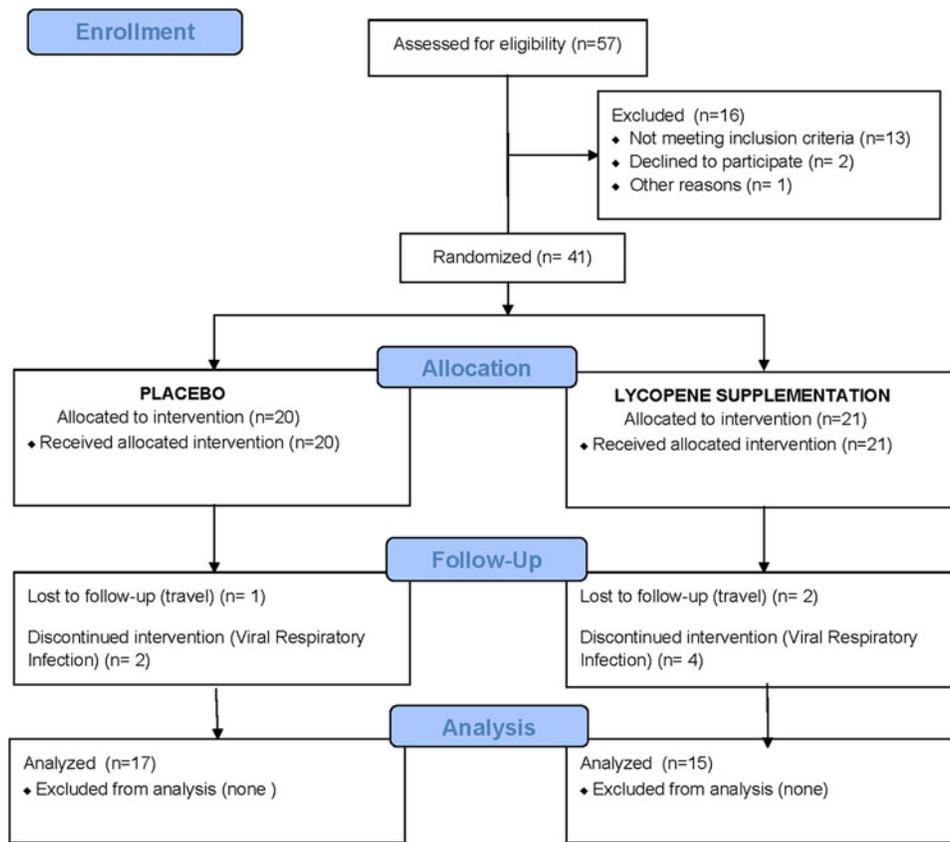


FIG. 1. Consort diagram.

immediately fixed with methanol, air-dried, and coded to provide sample anonymity for blinded analysis. All specimens were stored at  $-20^{\circ}\text{C}$  until the assay was performed. Smears were collected by the same technician to ensure uniform and consistent procedure.

#### Lycopene measurement

Lycopene measurements in serum were performed four times for each participant: before the run-in period, at day “0” of the trial (after run-in period), as well as at intermediate (after 2 weeks), and end (after 4 weeks) points of the trial. Lycopene concentration in all serum samples was measured using an HPLC protocol with modifications.<sup>(19)</sup> In brief, 400  $\mu\text{L}$  of serum was mixed with 400  $\mu\text{L}$  of ethanol and extracted twice with 2 mL hexane. The combined hexane layers were evaporated to dryness under vacuum (Scan Speed 32 centrifuge) and the residue reconstituted to 100  $\mu\text{L}$  in sample solution (absolute ethanol–methylene chloride, 5:1, v/v). The specimens were centrifuged again (15 minutes at 10,000 g), and the supernatant was transferred to HPLC vials.

Five microliters of the extract was injected into an Acquity HSS T3 75  $\times$  2.1 mm 1.8  $\mu\text{m}$  column (Waters, MA) preceded by an Acquity HSS T3 1.8  $\mu\text{m}$  VanGuard precolumn (Waters) and eluted isocratically at  $45^{\circ}\text{C}$  with the mobile phase (acetonitrile–0.08% phosphoric acid solution–tert-butyl methyl ether, 70:5:25, v/v/v) at a flow rate of 0.5 mL/min. The peaks corresponding to lycopene isomers were detected by a Photodiode Array Detector (Waters) at 474 nm. The peak areas were measured

using Empower 3 software (Waters). The lycopene concentration in serum samples was calculated by reference to an analytical standard (lycopene from tomato, L9879; Sigma).

#### IF analysis of facial skin smears

The smear specimens were fixed with methanol and permeabilized with Triton X-100. The processed specimens containing corneocytes and sebum remnants were stained using a direct IF protocol with fluorescein isothiocyanate-conjugated mAb (5  $\mu\text{g}/\text{mL}$ ) against lycopene recently developed and reported by our group.<sup>(16)</sup> Fluorescent staining was performed according to the basic IF protocol<sup>(20)</sup> and observed using a Nikon Eclipse 50i microscope with fluorescence attachment. The semiquantitative analysis was based on visual assessment of fluorescence levels in corneocytes and surrounding sebum in 20 random fields of view at 200 $\times$  magnification.

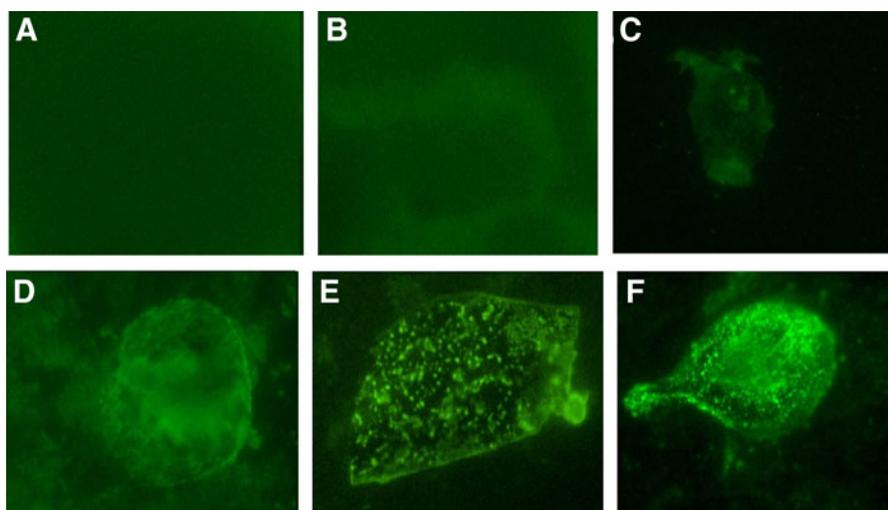
The scoring system employed with arbitrary images is shown in Figure 2. Fluorescence intensity in the samples was arbitrarily classified on a 6-point ordinal scale ranging from “0” to “4” as follows:

Score “0”—no fluorescence in the field; (Fig. 2A).

Score “0.5”—traces of fluorescence in the field; (Fig. 2B).

Score “1”—weak fluorescence inside some corneocytes and sebum remnants; (Fig. 2C).

Score “2”—moderate fluorescence inside of some corneocytes and sebum remnants; (Fig. 2D).



**FIG. 2.** Arbitrary images of corneocyte IF. Facial skin smears were obtained from the participants, stained with lycopene-specific labeled mAb, and analyzed under fluorescent microscope as described in the Material and Methods section. A panel of arbitrary images (A–F) used for evaluation of IF intensity in each individual specimen is shown. IF, immunofluorescence; mAb, monoclonal antibody.

Score “3”—strong fluorescence of some cells or areas of sebum background; (Fig. 2E).

Score “4”—extremely strong fluorescence (confluent fluorescence of sebum remnants and majority of corneocytes); (Fig. 2F).

Fluorescence assessment of each sample was rigorously performed and repeated three times by an independent morphologist who was not aware of specimens belonging to study groups. A comparison of IF results and serum lycopene concentrations was performed by a statistician and the principal investigator with the assumption that HPLC detection of lycopene in serum was a reference value.

#### Statistical analysis

All quantitative results reflecting enrollment data and randomization as well as arbitrary IF values are shown as averages with standard deviations. Results for lycopene quantification in serum are shown using descriptive statistical values (mean, standard deviation, median, and range values as well as 95% confidence intervals [CIs]). Paired *t*-test (two-sided *p*-values calculated) was applied to determine statistical significance for the differences between time points. *t*-Test for independent means was used for comparisons between groups. All data handling and statistical analyses were performed using IBM SPSS 19.0 statistical package (IBM, Inc., Armonk, NY).

## Results

### Randomization

Table 1 shows the baseline characteristics of the two major groups of the study (placebo vs. lycopene supplementation group) at the randomization time point. Each enrolled individual matched the inclusion/exclusion criteria parameters set in the study protocol. Therefore, all averages for baseline values were in the acceptable range required by the study protocol. No significant differences were seen in the gender identity, BMI values, pulse/blood pressure values, and basic

biochemistry values between the two groups, suggesting acceptable randomization of the volunteers and uncompromised health status. Most importantly, volunteers from both groups showed no statistical difference in serum lycopene level at the randomization time point of the trial (Table 1).

### Serum lycopene levels

However, there were some variations in serum lycopene concentration for both groups of the study at the enrollment phase of the trial, reflecting possibly the individual differences

**TABLE 1. BASELINE CHARACTERISTICS OF THE ENROLLED VOLUNTEERS**

<i>Parameters</i>	<i>Placebo</i>	<i>Lycopene supplementation</i>
Number of patients	17	15
Males	8	9
Females	9	6
Age	36.12 ± 1.62	34.66 ± 2.01
Light/moderate smokers	5	6
Body mass index	21.34 ± 1.64	20.39 ± 1.33
AST in U/L	31.08 ± 2.63	26.22 ± 5.39
ALT in U/L	24.33 ± 1.77	30.7 ± 4.01
Fasting glucose in mmol/L	5.31 ± 0.33	5.50 ± 0.21
Total cholesterol in mg/dL	140.33 ± 12.71	127.65 ± 14.76
Triglycerides in mg/dL	110.98 ± 8.64	115.43 ± 6.97
LDL cholesterol mg/dL	108.75 ± 6.55	114.73 ± 4.31
Pulse rate per minute	69.58 ± 5.44	73.78 ± 3.65
Blood pressure in mm Hg		
Systolic	110.43 ± 4.66	113.21 ± 3.58
Diastolic	72.94 ± 4.21	69.56 ± 3.98
Serum lycopene nM	151.58 ± 31.10	161.73 ± 43.21

Averages with standard deviations.

The volunteers were screened against inclusion/exclusion criteria, underwent physical and laboratory investigation, and were randomized as described in the Materials and Methods section. The results at the randomization time point of the trial are shown.

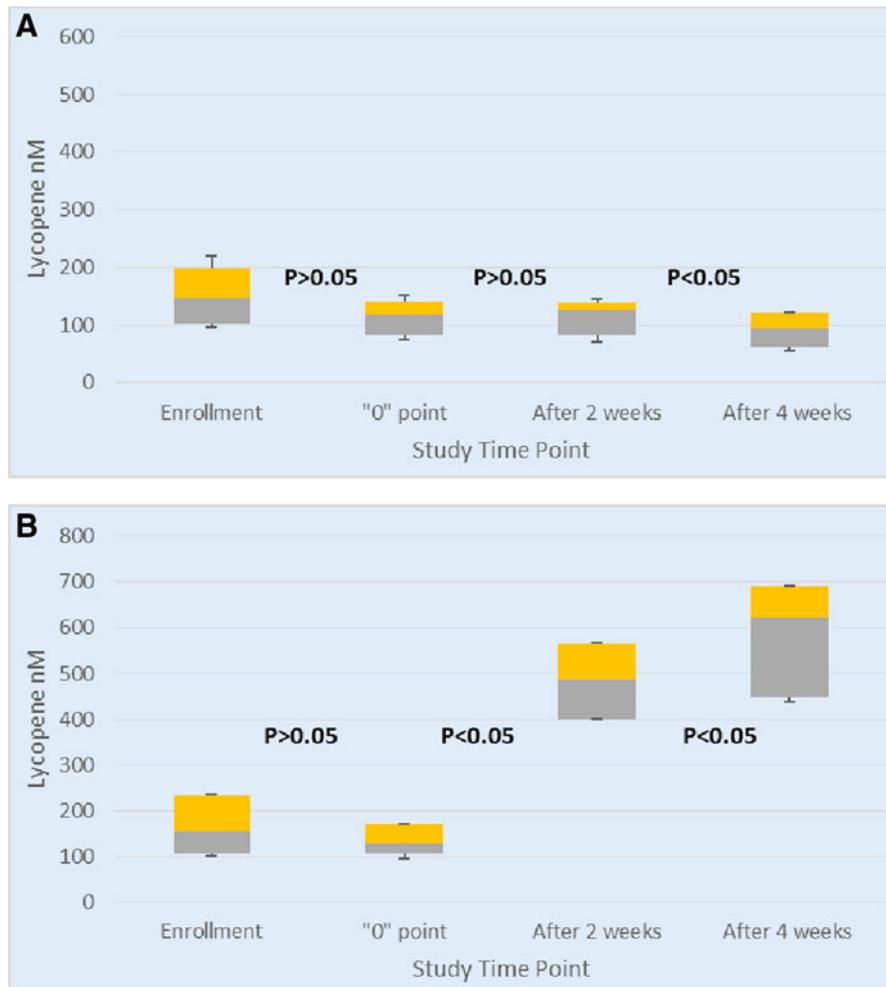
ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL, low density lipoprotein.

in nutritional status of the volunteers (Fig. 3A and B). These variations had been minimized at the “0” time point of the study after the run-in period, suggesting that restricting intake of lycopene-containing food products during the run-in period was effective at levelling out circulating lycopene values. As exemplified by the placebo group results, the median value of serum lycopene concentration at the randomization time point was 147.0 nM (95% CIs: 199.2/103.0). However, after the run-in period, these values occupied a lower and narrower statistical range of 118.0 nM (95% CIs: 139.8/83.0). A similar pattern was seen in the lycopene supplementation group after the run-in period (Fig. 3B). Importantly, there was no gender-related difference in serum lycopene levels at the enrollment phase of the trial or at any time point of the trial thereafter ( $p > 0.05$ , results not shown). As can be seen from Figure 3A and B, lycopene supplementation gave a significant increase in circulating lycopene levels. The median values for this parameter increased from 128.0 nM (95% CIs: 170.5/106.6) at the “0” time point to 565.8 nM (95% CIs: 565.8/399.0,  $p < 0.05$ ) and 690.2 nM (95% CIs: 499.9/693.0,  $p < 0.05$ ) after 2 and 4 weeks of supplementa-

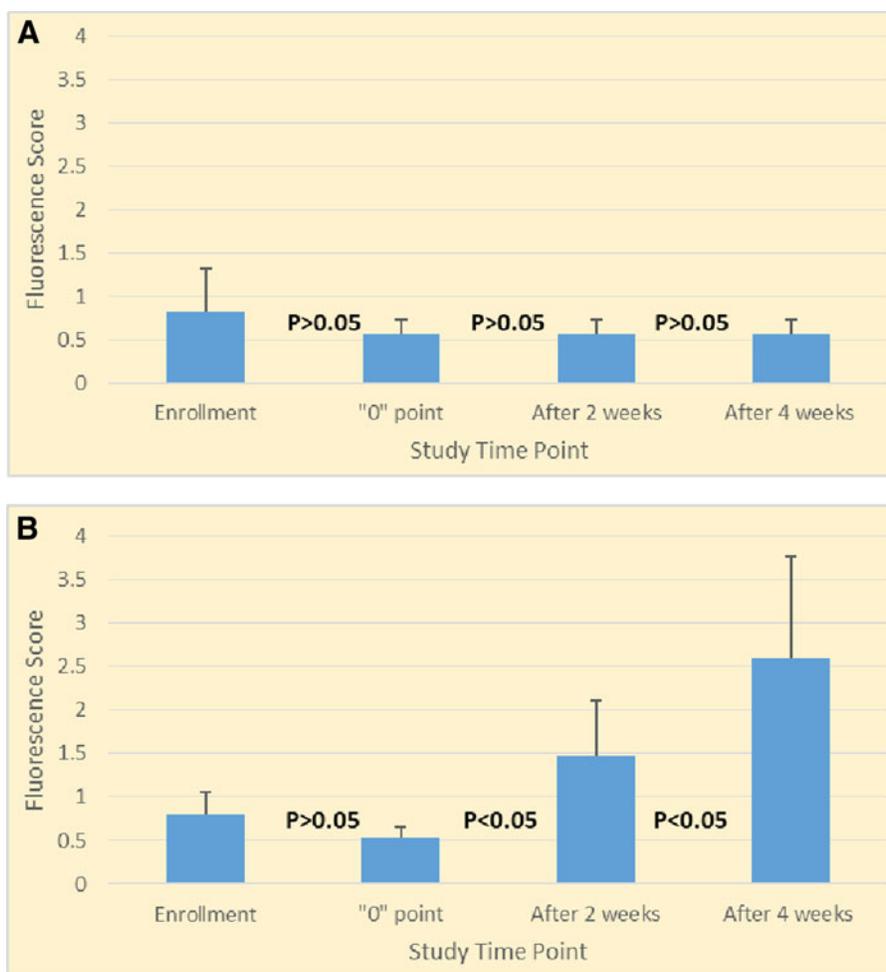
tion, respectively (Fig. 3B). Notably, the serum lycopene content in the placebo group was unchanged after 2 weeks of the study ( $p < 0.05$ ) and slightly declined after 4 weeks of placebo intake from a median value of 125.0 nM (95% CIs: 138.0/83.0) at the “0” time point to 94.0 nM (95% CIs: 121.0/62.2) at the end of the study with borderline statistical significance ( $p = 0.056$ ).

#### Skin smears

IF analysis showed that there were quantifiable changes in immunostaining of the skin smears, in particular corneocytes and sebum, collected from the volunteers. As can be seen from Figure 4A and B, the volunteers from both study groups had similar arbitrary scores for lycopene-specific IF staining at the enrollment stage of the trial. The variations in IF score values were somewhat minimized after the run-in period for both groups, which resembles the pattern of change seen for serum lycopene concentration. The placebo group was characterized by consistently low arbitrary IF scores for lycopene-specific IF staining at all time points of the study



**FIG. 3.** Changes in serum lycopene levels. The volunteers were screened against inclusion/exclusion criteria, underwent physical and laboratory investigation, and were randomized as described in the “Materials and Methods” section. Serum lycopene level was measured in volunteers from the “Placebo” group (A) and the “Lycopene Supplementation” (7 mg daily dose) group (B) after 2 and 4 weeks of the study and plotted against values for lycopene serum level measured in the volunteers at the enrollment phase and “0” time point of the study.



**FIG. 4.** IF score for skin smears. The volunteers were screened against inclusion/exclusion criteria, underwent physical and laboratory investigation, and were randomized as described in the “Materials and Methods” section. IF score for corneocytes and sebum was determined for skin smears obtained from volunteers in the “Placebo” (A) and “Lycopene Supplementation” (7 mg daily dose) groups (B) after 2 and 4 weeks of the study. Scores were then plotted against IF score for skin smears obtained from the volunteers at the enrollment stage and “0” time point of the study.

with average values for the arbitrary IF score of around 0.5. However, there was a statistically significant increase in IF score for volunteers supplemented with lycopene (Fig. 4B). After 2 weeks of lycopene supplementation, the average IF score for skin smears had risen to  $1.46 \pm 0.63$ , whereas 4 weeks supplementation was accompanied by an increase to  $2.60 \pm 1.16$  versus the “0” time point control value of  $0.53 \pm 0.12$ . Most of the IF signals seen in the skin smears at both time points originated from corneocyte inclusions, whereas fluctuations in sebum IF signal were more diffuse and modest.

#### Inter-rater reliability

There was reasonable agreement between IF scoring and serum lycopene concentration in the volunteers, especially in the lycopene supplementation group. There was 86.6% agreement in the set of paired variants for the intermediate time point and 80.0% agreement at the end point of the study between skin smear IF scores and circulating lycopene with the assumption that serum lycopene level is an arbitrary standard. Intraclass correlation coefficient between paired

values in the lycopene supplementation group was +0.49 at the 2-week supplementation time point and +0.63 at the end point of the study. All discordant observations (3 pairs out of 15 total paired sets) in the lycopene supplementation group were positive by means of lycopene increase in serum that were not supported by IF results in skin smears. The data set for the placebo group had a much less conclusive inter-rater reliability due to low numerical values for arbitrary IF scores and serum lycopene levels that barely changed during the course of the study.

#### Discussion

There is a growing body of scientific evidence that overall carotenoid consumption and blood lycopene level, in particular, are negatively associated with the prevalence and outcome of CVD, type 2 diabetes, and certain types of neoplastic growth and ophthalmological disorders.<sup>(2,7,9,21)</sup> Moreover, carotenoid bioavailability is reduced in aging persons and in persons with metabolic syndrome and obesity. Indirect assessment of carotenoid supplementation status in

the general population by food questionnaires and attempts to create the European carotenoid intake database based on habitual dietary intake<sup>(22,23)</sup> are opposed by reports suggesting low intestinal bioavailability of carotenoids.<sup>(9,14)</sup> Thus, there is a need for affordable and preferably noninvasive methods, allowing direct assessment of carotenoid supplementation status in the general population on a reasonably large scale.

As we have already reported, a direct IF protocol with lycopene-specific mAb allows a semiquantitative measurement of lycopene in corneocytes and sebum obtained from skin swabs. To the best of our knowledge, this observation is the first report identifying lycopene in skin tissue constituents. However, this finding aligns well with current knowledge of lycopene turnover and transport *in vivo*. Lycopene is a highly nonpolar and amphiphilic compound that can be transported and distributed among the tissues only after incorporation into lipoprotein particles—chylomicrons, very low density lipoprotein, and low density lipoprotein (LDL).<sup>(4)</sup> Upon absorption, lycopene can be identified in various tissues and organs—liver, adrenals, and prostate, which are characterized by high expression of LDL receptors and scavenger receptors.<sup>(4,24)</sup> In this regard, it should be mentioned that keratinocytes, a cellular predecessor of corneocytes, and corneocytes themselves, express a substantial number of LDL receptors and class B scavenger receptors.<sup>(25)</sup> Moreover, skin corneocytes are embedded in a highly ordered lipid lamellar phase, which promotes constant lipid trafficking in the stratum corneum and is essential for sebum formation.<sup>(26)</sup> This explains why lycopene content in cellular components of the epidermis and sebum may be highly reflective of overall lycopene supplementation status. Indeed, as we have already shown, oral intake of lycopene leads to a significant and time-dependent increase in serum lycopene concentration as well as to a concordant and quantifiable step-wise increase in lycopene-specific immunostaining in corneocytes and sebum. Moreover, there was a congruent tendency toward reduction of median values reflecting serum lycopene concentration and IF score after the run-in period when restricted lycopene intake was imposed on both groups of the study. And finally, placebo ingestion was accompanied by comparably low values for both serum lycopene level and IF score in the skin swabs.

Furthermore, there was a comfortably good statistical agreement between HPLC data and IF analysis. Intraclass correlation between paired values for the lycopene supplementation group was +0.49 at the 2-week supplementation time point and +0.63 at the end point of the study. All discordant observations (3 pairs out of 15 pairs) in the lycopene supplementation group were positive by means of lycopene increase in serum, which was not supported by IF results. Apart from being inexpensive, the IF assay is easy to conduct and interpret, with a turnaround time of around 3 hours.

At the same time, our study had some limitations. A larger cohort of volunteers would be helpful for evaluation of interrater reliability of the assay. It should also be clarified that the main purpose of this study was to establish the possibility of detecting lycopene in skin tissue by using a basic IF assay protocol with the lycopene-specific mAb. Only after that was the objective to evaluate the relationship between lycopene levels in skin samples with serum lycopene concentration. Vigorous optimization and standardization of the lycopene IF

assay for skin swabs is obviously required to delineate the advantages and limitations of the assay before introducing this method into laboratory practice of nutritional science. Finally, the results reported open the door to possible development of enzyme-linked immunosorbent-based assays for the detection of lycopene and possibly other carotenoids in skin swabs.

#### Author Disclosure Statement

No competing financial interests exist.

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