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Modulation of Cutaneous Carotenoid Content via Ozone Exposure

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Abstract: Ozone (O_3) is a harmful air pollutant to which we are constantly exposed. Given its strong oxidizing effects and pervasiveness in the air we breathe, O_3 is especially damaging to target organs in the respiratory system (e.g., lungs) and the integumentary apparatus (e.g., skin). Both of these systems act as a barrier and are able to limit the penetration of atmospheric pollutants into the body. In this regard, skin—the largest and main barrier against atmospheric intrusions—offers continuous protection against environmental intrusions. The skin is equipped with several defensive molecules that act as protective intracellular antioxidants against oxidative intrusions, including O_3. Among these antioxidants are carotenoids, a family of lipophilic phytonutrients that are abundant in fruits and vegetables. It is well established that carotenoids accumulate in the epidermis layer of the skin, where they confer protection against oxidative intrusions and modulate inflammation, and that there is a direct correlation between skin and serum carotenoids level. The present study aimed to evaluate the variations in carotenoid content present in human skin prior to and after O_3 exposure in 141 human subjects. Carotenoids were measured non-invasively using a resonance Raman spectroscopy (RRS)-based photonic device (Pharmanex BioPhotonic Scanner (BPS) Nu Skin Enterprises). In each volunteer, RRS skin carotenoids were determined at baseline and after 15 and 30 min of exposure to O_3 0.8 ppm. The data obtained have an indicative value for individual variations in the cutaneous carotenoids, which have been shown to correlate with plasmatic contents. After the first 15 min of O_3 exposure, there was a modulation of skin carotenoids, confirming their importance in the maintenance of cutaneous redox homeostasis.

Keywords: skin barrier; carotenoids; ozone exposure; Raman spectroscopy; statistics

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1. Introduction

Environmental pollution means the presence of polluting elements in the whole environment, from the atmosphere to water and soil [1]. The imbalance in the ecosystem resulting from the release of these substances into the environment often causes irreversible damage to the health of all living beings, that is, plants and animals, including humans.

Excluding the very few causes of natural pollution, such as sulfur and carbon dioxide emissions caused by volcanic eruptions, human activity is the primary source of environmental pollution [2]. Air pollution is a local, pan-European, and pan-hemisphere problem. Air pollutants emitted in one country can be carried into the atmosphere, contributing to or resulting in poor air quality elsewhere.

Particulate matter, nitrogen dioxide, and ground-level Ozone (O_3) are considered the three pollutants that most significantly affect human health. About 90% of city dwellers are exposed to concentrations of pollutants above air quality levels that are considered harmful to health. Photochemical smog, commonly known as "summer smog", has caused respiratory problems in the European population for several decades and is a possible source of serious damage to plants.

Summer smog is formed by photochemical reactions involving numerous gases present in the troposphere, the layer of atmosphere between the Earth's surface and an altitude of 7–15 km. The main precursors are nitrogen oxides (NOx, i.e., NO and NO_2), volatile organic compounds (VOC), methane (CH_4), and carbon monoxide (CO). Many human activities give rise to these pollutants, such as the use of fossil fuels, especially for transportation, and the use of products containing organic solvents.

Sunlight, by acting on precursor pollutants, causes the formation of a range of compounds known as photochemical oxidants. The most important photochemical oxidant, because of its abundance and toxicity, is ozone (O_3). Threshold values for O_3 concentrations, set in order to protect human health, vegetation, and ecosystems, are frequently exceeded in most European countries. Ozone is a gaseous molecule composed of three oxygen molecules present both in the stratosphere (with a protective effect against UV rays) and in the troposphere (at ground level) [3]. Tropospheric O_3 is not emitted directly into the air but is formed as a result of chemical reactions between other environmental pollutants, in particular between nitrogen oxides (NOx) and volatile organic compounds (VOCs) in the presence of sunlight. While ozone production is greatest in urban areas on hot sunny days, it can still reach unhealthy levels in the colder months. Additionally, ozone can be transported over long distances by wind and weather systems, adversely affecting individuals and communities in suburban and rural areas far from the original source of its formation. Tropospheric O_3 is particularly dangerous for humans, as it is ubiquitous in the environment and is associated with several related inflammatory diseases [4]. At low concentrations (0.1–0.2 ppm), this gas is capable of causing irritation to the throat and eyes, while exposure to higher concentrations (0.8–1 ppm) leads to irritations that extend to the entire respiratory system, causing coughing, chest tightness, asthma attacks, and respiratory problems [5–7]. In addition to the respiratory tract, there is strong evidence that the skin is a vulnerable target for ozone damage [8–10]. In fact, the skin is the main biological barrier that defends our bodies from the external environment. A general mechanism by which O_3 affects the skin is through the generation of bioactive molecules (aldehydes and H_2O_2) derived from its pro-oxidant properties, leading to oxidative damage, including lipid peroxidation [11–13]. It must be said that the skin has an effective defensive capacity against oxidative damage thanks to both the enzymatic machinery and micronutrients derived from one's diet [14]. Carotenoids are a family of lipophilic phytonutrients abundant in fruits and vegetables that protect against oxidative and inflammatory stress in the skin. Of the more than 600 naturally occurring carotenoids, lycopene, β-carotene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin, phytoene, and phytofluene are the primary carotenoids detected in human skin [15,16].

Interestingly, lycopene, a carotenoid that is abundant in tomatoes and tomato products such as sauces and pastes, is the most abundant carotenoid present in human skin. Carotenoids are known to accumulate in the outermost layer of the epidermis, the stratum corneum, where they confer protection against oxidative intrusions and modulate inflammation. For example, carotenoids such as α-, β-, and γ-carotene and β-cryptoxanthin have provitamin A activity. Carotenoids have the unique ability to quench singlet oxygen and can also act as "chain-breaking" antioxidants, reducing peroxyl radicals and protecting plasma membranes against lipid peroxidation. For instance, it has been suggested that a molecule of beta-carotene is able to neutralize about 1000 molecules of singlet oxygen.

The carotenoids are not only synthesized in plants and in photosynthetic organisms but also in some non-photosynthetic organisms, such as bacteria, yeasts, and molds [17]. As humans are not able to synthesize carotenoids, plasma and tissue levels reflect carotenoid intake and bioavailability from food sources, primarily colorful fruits and vegetables. Since carotenoids are fat-soluble, their bioavailability is limited in the absence of dietary fat. For example, the addition of olive oil to tomatoes during cooking significantly increases lycopene plasma levels compared with tomatoes cooked without olive oil [18]. Individuals who consume a diet rich in colorful fruits and vegetables typically have higher levels of serum and tissue carotenoids. As they are predominantly deposited in the stratum corneum, the transfer of carotenoids to the skin is hypothesized to occur either via diffusion from the blood and adipocytes or transportation through sebaceous and eccrine sweat glands. Following oral consumption, this process is reported to take between 1 and 3 days. Lifestyle factors, including sun exposure without the use of sunscreen, being overweight, obesity, heavy exercise, and cigarette smoking—all of which are associated with increased oxidative stress—are known to deplete carotenoids in humans [19]. While many lifestyle factors are modifiable, exposure to environmental pollutants is unavoidable [20]. Therefore, the purpose of the present study was to investigate the effects of acute ozone exposure, an environmental toxicant, on cutaneous b-carotene and lycopene levels [15,16]. Carotenoids were assessed in the skin of 141 volunteers, male and female, aged between 18 and 68, using a non-invasive resonance Raman spectroscopy (RRS)-based photonic device (Pharmanex BioPhotonic Scanner (BPS) Nu Skin Enterprises). In recent years, skin carotenoid scores (SCSs) assessed using RRS have been increasingly evaluated as a potential alternative biomarker for skin carotenoids and fruit and vegetable intake.

2. Materials and Methods

2.1. Skin Carotenoid Quantification

The instrument is a Raman radiation emitter/scanner, patented for the quantification of carotenoids present in human skin, a parameter equivalent to the concentration of carotenoids circulating in plasma [21]. The carotenoids are excited with low-intensity pulsed radiation (471.3–473 nm) emitted by LED diodes. The scattered light is detected at 507.8–509.8 nm by the scanner, which converts the Raman intensity into counts (the skin carotenoid score (SCS)), expressed in thousands of units increasing according to the content of the excited carotenoid molecules.

A computer then transforms the scanner signals into a colored scale ranging from red (poor carotenoid score, SCS < 20,000) to dark blue (high carotenoid score, SCS > 90,000). The system is continuously tracked by an application installed on an Apple iPad connected via Bluetooth to the scanner.

The SCS can be converted into cutaneous carotenoid quantitative data for the patient, expressed in micrograms/milliliters using the following equation:

$$Y = 12703\ X + 5891.7\ X = \frac{Y - 5891.7}{12703}$$

where "Y" is the SCS value, and "X" is the concentration of carotenoids expressed as micrograms/mL of serum (Figure 1) [18,19]. The correction factor, 5891.7, indicates the skin absorption provided by the other photosensitive molecules present in the dermis (collagen, porphyrin, and hemoglobin). The normal range of beta-carotene in the blood is 50 to 300 mcg/dL or 0.93 to 5.59 micromol/L.

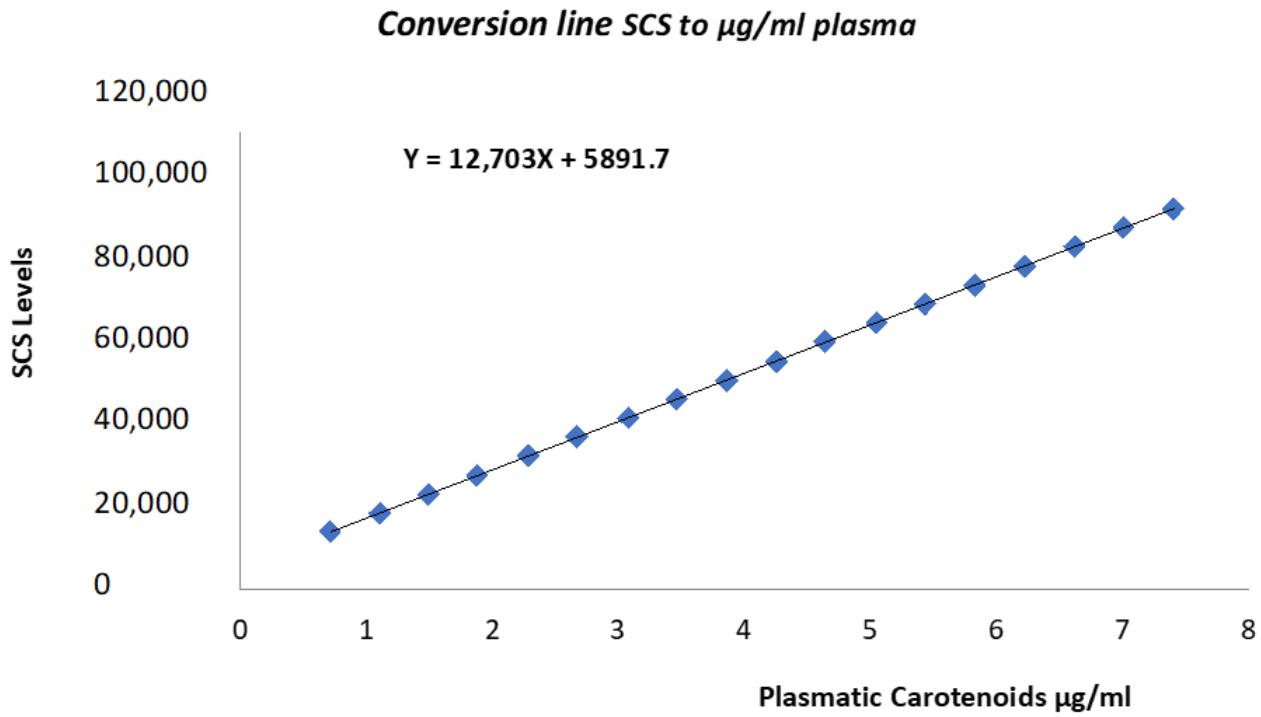

Figure 1. Conversion line of the SCS in µg/mL of plasma. The values expressed on the line make it possible to determine the value of carotenoids in µg/mL in the plasma. For example, a 25,000 SCS is equivalent to 1.5 µg/mL, and hence, a 1000 SCS corresponds to 0.06 µg/mL plasma.

2.2. Ozone Exposure

O_3 was generated from O_2 using an electrical corona arc discharge (Model 306 ozone Calibration Source, 2B Technologies, Ozone Solution, 117th Avenue Broomfield, CO 80020, USA), as previously described [20]. Briefly, the O_2–O_3 mixture (95% O_2, 5% O_3) was combined with ambient air and allowed to flow into a Teflon-lined exposure chamber. The O_3 concentration in the chamber was adjusted to varying ppm outputs and continuously monitored using an O_3 detector. Hands and forearms were directly exposed to O_3 inside a plexiglass box (Figure 2). Temperature and humidity were monitored during exposures (25 °C and 45–55%, respectively) [22–24].

The study was performed on 141 healthy volunteers over a period of six months, from December 2017 to June 2018. A local independent institutional review board approved the study protocol. All subjects participating in this trial gave their written, informed consent.

The measurement of cutaneous carotenoids on the palm of the hand was performed at three consecutive timepoints:

Time zero, before inserting the arm inside the ozone chamber;

Time 15 min, after an intermediate exposure for 15 min to ozone;

Time 30 min, at the end of the exposure, determined by the protocol.

Ozone was maintained in the exposure chamber at a concentration of 0.8 ppm, equivalent to the concentrations of this gas on days with the highest concentrations of pollutants present in the atmosphere in cities with a high rate of vehicular traffic or present in rural environments on days with high solar irradiation and poor ventilation [25].

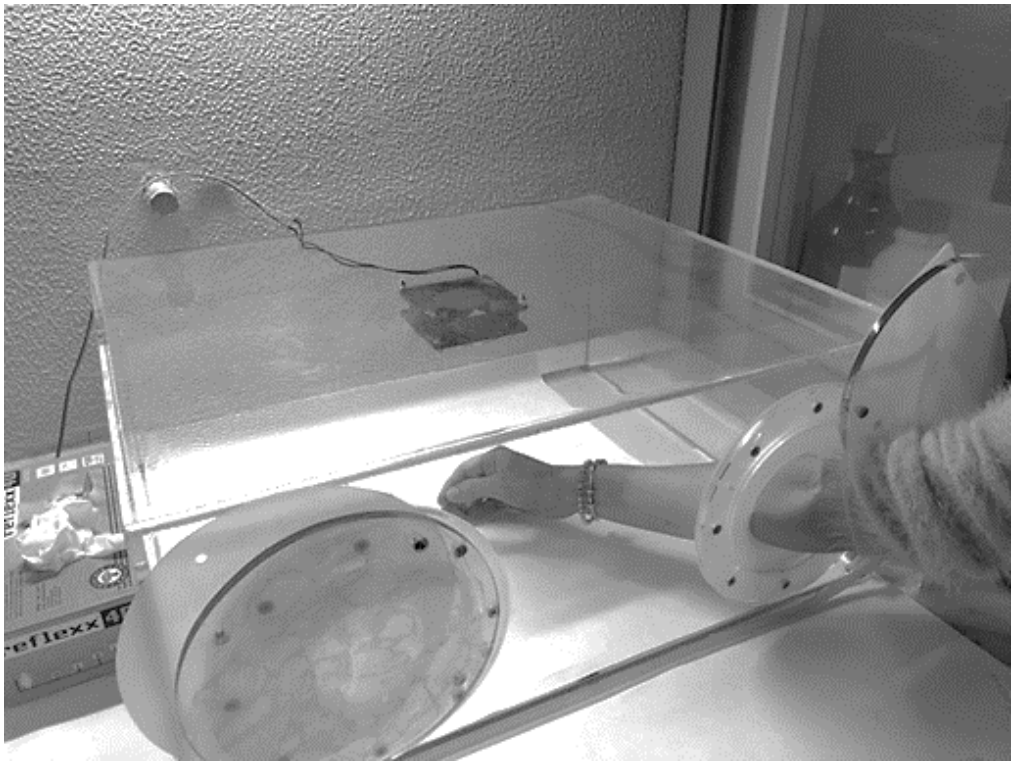

Figure 2. Ozone exposure chamber.

3. Results

3.1. Cutaneous Carotenoid Content

The variation in the cutaneous carotenoid content in the subjects during ozone exposure was calculated and is shown in Figure 3 at the beginning (T0); intermediate time point (T15 min); and at the end of the exposure (T30 min). The data collected as a percentage indicate that, in a plurality of samples (29.5%), carotenoid levels decreased after 15 min and then rose again after 30′ of exposure. In contrast, in 24.5% of the samples, there was an increase after 15 min and a decrease after 30 min. In 20% of subjects, the levels decreased at both timepoints, 15 min and 30 min, while in 17% of subjects, skin carotenoids increased after 15 min and after 30 min. Finally, only in 2.5% of subjects did the levels remain stable during all experimental procedures.

The cutaneous carotenoid contents before exposure to ozone (T0), depended on the sex, BMI, exercise practices, and cigarette smoking habits of the analyzed subjects. The *t*-test was verified with the normality of distribution, verified with the Kolmogorov–Smirnov method. Table 1.

3.2. SCS Variations in Relation to the Portions of Fruits and Vegetables Consumed

The relationships between the portions of fruits and vegetables consumed daily and the levels of skin carotenoids score were considered. As shown in Figure 4, the SCS significantly increased based on the number of fruit and vegetable servings.

3.3. Variation in Carotenoids on the Whole Group after 15 min of Ozone Treatment

As described in Figure 5, after the first 15 min, the amplitude variations were directly proportional to the basal levels of the subjects; in fact, the higher the number of carotenoids present at the beginning (T0), the higher the variation, both positively (Figure 5A) and negatively (Figure 5B).

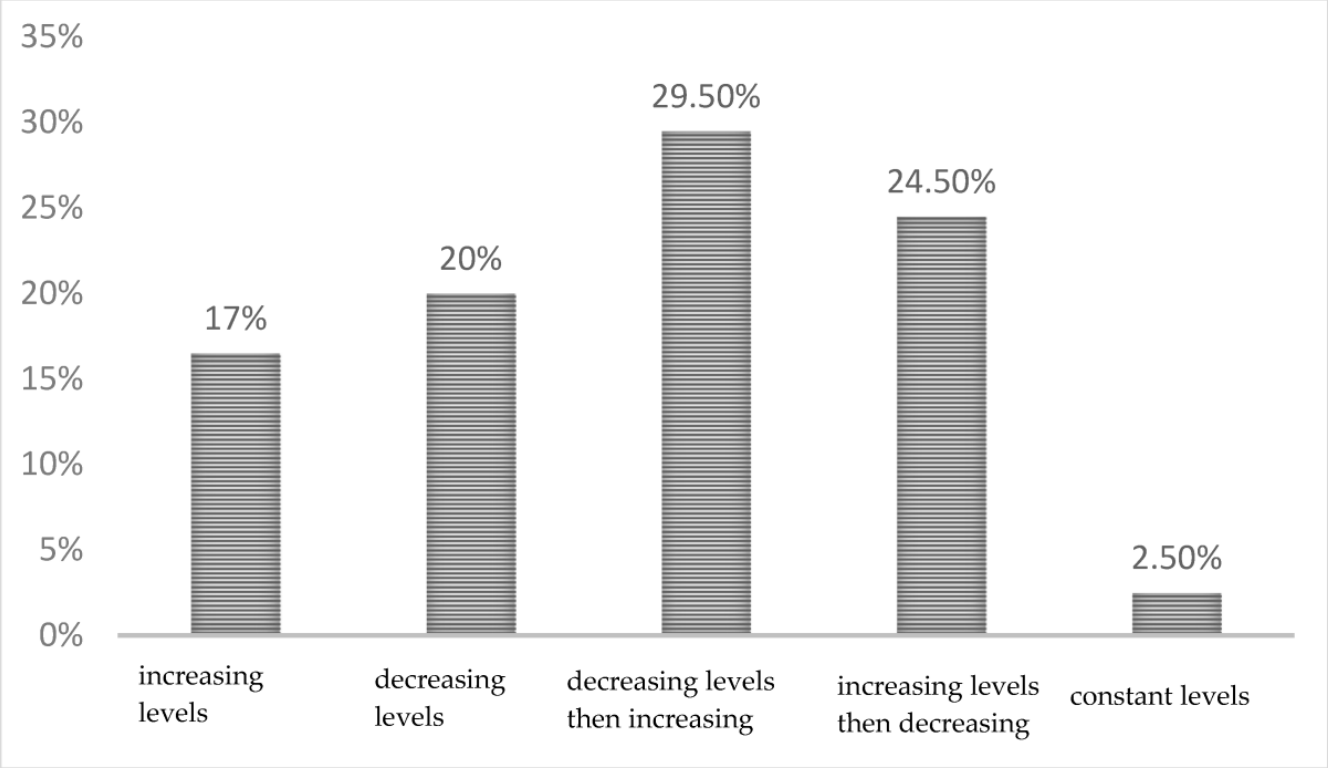

Figure 3. Variation in the carotenoid content of the skin of all subjects during ozone treatment: at the beginning (T0); halfway (T15′); and at the end (T30′) of exposure.

Table 1. SCS (skin carotenoid score) averages: Data are represented as mean ± D.S. of SCS at T0 based on sex; body mass index (BMI), smoking, and exercise. Statistical analysis was performed with a *t*-test. Significant at a *p*-value of <0.05.

	N (%)	T0 h SCS (Average ± DS)	*p*-Value
Gender			
Male	32	48,288 ± 13,667	N.S.
Female	68	50,395 ± 15,487	
BMI			
Normal weight	86	49,032 ± 15,614	$p < 0.05$
Overweight	14	49,066 ± 12,452	
Smoker			
Yes	21	43,181 ± 14,555	$p < 0.05$
No	79	50,411 ± 14,977	
Exercise			
Yes	44	46,334 ± 10,182	$p < 0.05$
No	56	50,950 ± 17,075	

3.4. Variation in SCS Values Following Ozone Treatment in the Smoker and Non-Smoker Groups

In relation to the habit of cigarette smoking in the tested subjects (Figure 6), the trend in dermal carotenoid contents after 15 min of ozone exposure was found to wane in smokers (Figure 6A) and increase in non-smokers (Figure 6B). While in smokers the increase in SCS was only 32%, in non-smokers, the increased SCS levels after 15′ further increased by 23%. In both cases, the *t*-test results were statistically significant ($p < 0.05$).

3.5. Variations in Expected Carotenoids Based on the Consumption of Fruits and Vegetables

The expected prospective average of the variation in carotenoid content and SCSs at different timepoints of ozone exposure was calculated based on daily servings of fruits and vegetables consumed by the subjects. As shown in Figure 7, there was an increase in SCSs based on the number of daily servings of vegetables and fruits consumed, which is particularly relevant in subjects who consumed six servings of colored fruits and/or vegetables per day.

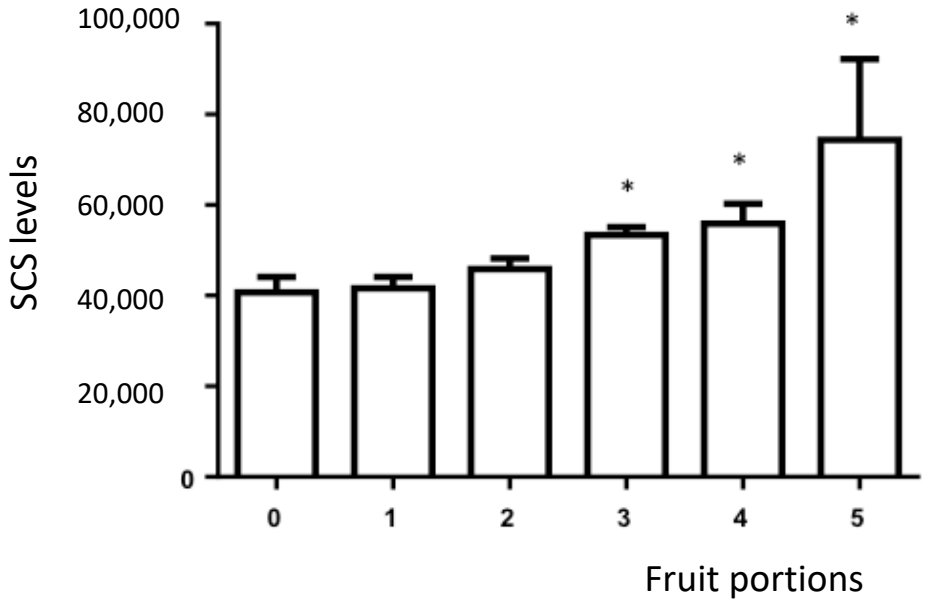

Figure 4. Daily portions of fruits and vegetables: the skin carotenoid score (SCS) was evaluated based on fruit and vegetable servings taken daily. Statistical analysis was performed with an ANOVA test followed by a post hoc Bonferroni test. * $p < 0.05$ vs. 5 fruit/vegetable portions.

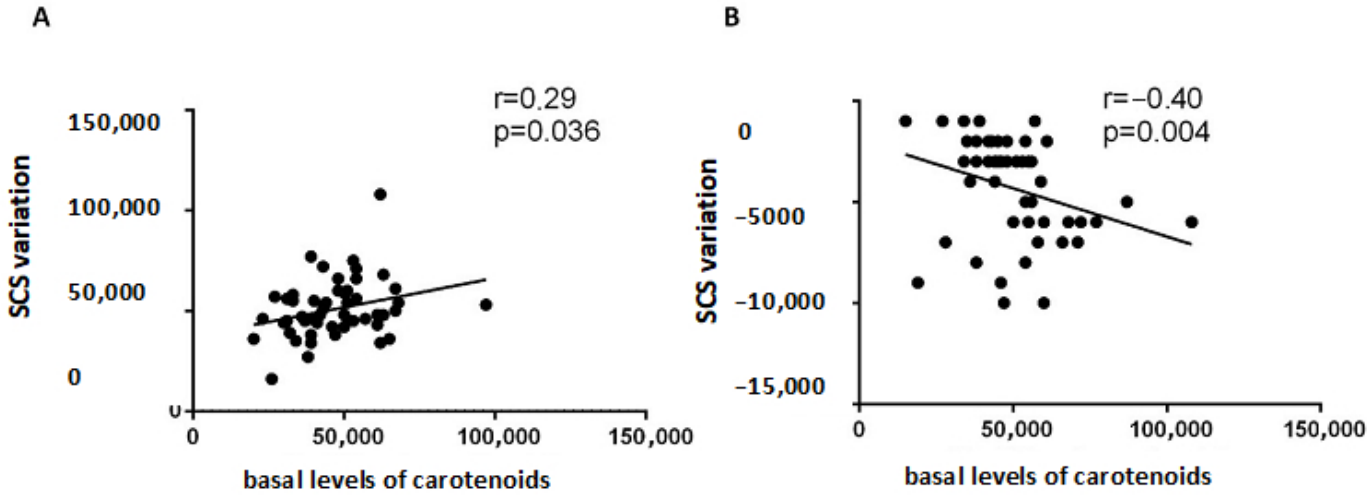

Figure 5. SCS variations. Absolute change in negative (**A**) and positive (**B**) carotenoid levels (measured at T0 and after 15 min of ozone exposure) in relation to the baseline levels of the samples analyzed.

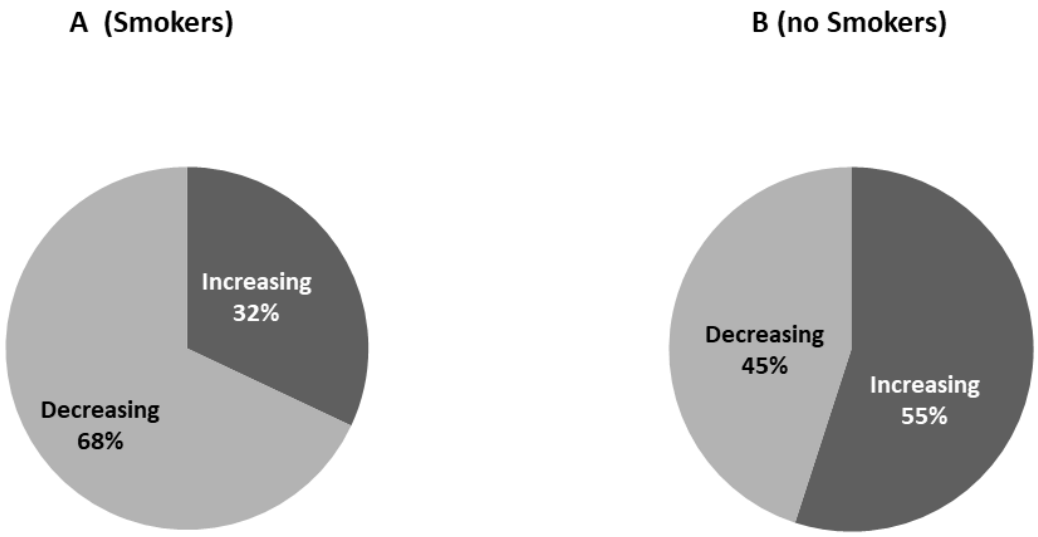

Figure 6. Trends in the content of cutaneous carotenoids after 15 min of ozone exposure in smokers (**A**) and non-smokers (**B**).

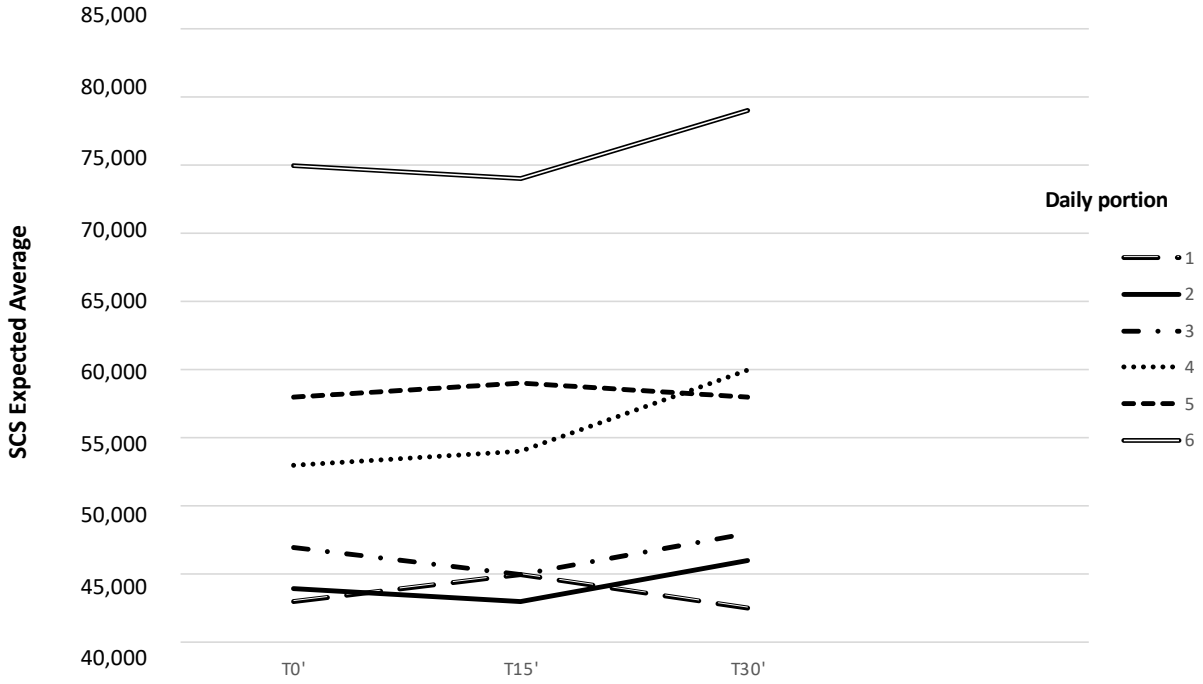

Figure 7. Average expected SCS (skin carotenoid score) levels at different times after ozone exposure in relation to the daily servings of fruits and vegetables consumed.

4. Discussion

The skin carotenoid score (SCS), measured using Raman resonance spectroscopy (RRS), can serve as a measure to evaluate changes in the concentration of skin carotenoids following dietary fruit and vegetable intake. Predominantly accumulated in the stratum corneum, skin carotenoids assessed with RRS have become increasingly valuable parameters as an index of the skin's health and cutaneous ability to counteract the oxidative intrusions derived from exposure to environmental pollutants such as ozone.

The reactive capacity of cutaneous carotenoids in contrasting environmental intrusions also seems to be related to various additional factors, such as the co-presence of other pollutants in the environment, lifestyles, smoking, and diet, the latter including the consumption of colored fruits and vegetables.

The data presented here are the result of a screening carried out on a sample of 141 healthy subjects. The purpose of the study was to understand skin carotenoid variations after O_3 exposure at concentration levels found in polluted cities.

Carotenoid concentration measurements were performed directly on the skin of the palm of the hand using a digital scanner (Pharmanex Bio Photonic Scanner) emitting pulsed Raman laser radiation of 473 nm. This radiation excites by causing vibrational motion; photosensitive molecules (among which, the carotenoids present in the skin) emit radiation at a higher energy level, 575 nm, and proportional to their concentration. This is recorded as a skin carotenoid score (SCS), converted into thousands of units and calculated based on the calibration line described in the Methods section.

This method of measuring carotenoids was recently validated by several researchers [26–28]. Indeed, the authors found a clear correlation between skin and serum carotenoid levels in both infants and adults by measuring over 300 healthy subjects. Therefore, it is possible to extrapolate that the skin carotenoid levels measured in our study could also represent the serum levels of the subjects.

The obtained results regarding the basal content of carotenoids showed that there was no statistical difference between males and females, as shown in previous work [29,30] and contrary to what was reported by S.T. Mayne et al. [31]. This discrepancy could depend on the different subject populations between the two studies since, in the work of S.T. Mayne et al., the male population was a bit older, and in the same study, the authors showed that carotenoid levels decreased with age. Smokers had significantly lower levels of carotenoid content ($p < 0.05$), with a difference between the mean of the two groups of 7230 SCS; this observation paralleled previous work by V. Ermakov et al., 2010 and 2018 [32–34]. Physically active people seem to favor the accumulation of carotenoids at higher levels than sedentary individuals [35,36]. It needs to be taken into consideration that the analyzed population was composed of very young individuals with very few overweight subjects; we believe that these data need to be confirmed in a more heterogenous population before linking overweight and carotenoid levels. Of note is the fact that the different levels of daily fruit and vegetable consumption clearly affect SCS levels in individuals, in that those who reported consuming five or more daily servings, compared with those who consumed less than three, had significantly higher carotenoid concentrations: 70,000 vs. 40,000, respectively.

Regarding the changes in SCS induced by O_3 exposure, we found that the amplitude of the change in carotenoid levels after the first 15 min of exposure was directly proportional to the baseline levels of the subjects; in fact, the higher the number of carotenoids present and available prior to exposure, the greater the change in SCS (both in negative and positive values). It appears that, in individuals with high baseline carotenoids, there is a more rapid cutaneous response to O_3 exposure, while individuals with low baseline SCS levels responded ineffectively to O_3.

It is generally suggested that the toxic effects of O_3 are mediated through free-radical reactions (although O_3 is not a radical species, per se), achieved either directly via the oxidation of biomolecules to produce classical radical species (hydroxyl radical) or driving the radical-dependent production of cytotoxic, non-radical species (aldehydes) [37].

Research from our group and others has shown the toxic effect of O_3 in cutaneous tissues; in particular, we were able to detect an increase in oxidation products (carbonyls and 4HNE) and a decrease in antioxidants defenses (vitamin E, vitamin C, GSH, and glutathione) [38,39]. Therefore, it is possible that carotenoids play an effective role in protecting the skin from O_3 damage and, more generally, oxidative damage.

In a previous "in vivo" study, we were able to show that a carotenoid-enriched diet prevented O_3-induced skin "oxinflammation" [40] in the skin of SKH1 mice [41]. In particular, the mentioned work showed that carotenoids were able to prevent the O_3 induction of HO-1, MIP-1, TNFa, and iNOS, all of which are related to inflammation and defensive mechanisms. These data correlate with several other studies where the ability of O_3 to activate NFkB, an inflammation redox transcription factor, was demonstrated.

In the present human study, we showed that, after 15 min of O_3 exposure, 56% of subjects had a clear decrease in their SCSs, presumably caused by the immediate availability of a sufficient quantity of carotenoids, quenching oxidative mediators formed upon ozone exposure (i.e., peroxidation).

In the remaining 44% of individuals, an increase in SCSs was detected after the 15 min exposure to O_3, and this could be a consequence of an induced redistribution of carotenoids from plasma to tissue [42,43].

Indeed, it has been proposed that the quantity of carotenoids in the skin is proportional to plasma; therefore, plasma plays a key role in restoring carotenoid levels once it is decreased by exogenous challenges, functioning as a reservoir [44–46]. This is in line with our results, where subjects with SCS baseline levels over 48,000 showed a decrease in carotenoid levels after O_3 exposure, while the group with SCS baseline levels lower than 48,000 showed an increase in SCS levels. This effect could indicate that subjects with a large number of carotenoids can promptly use them to restore their tissue levels, while subjects with a low baseline have a significant decrease in tissue carotenoid content. The association between SCS values and dietary carotenoids suggests the existence of individual variability in the circulation and deposition of carotenoids in the skin.

Considering all the data presented here—despite the limited number of recruited volunteers and the very homogeneous composition of the sample, most of whom were young and in good health—some interesting aspects are highlighted, including evident changes in cutaneous carotenoid levels in the first few minutes of O_3 exposure, confirming the importance of antioxidant content in the skin as a first defense against outdoor intrusions. Therefore, high carotenoid tissue content, which occurs beneficially through diet and a proper lifestyle, can help overcome oxidative stress induced by pollution exposure, including UV radiation.

In conclusion, the present study was able to assess that there was no statistically significant difference in carotenoid content between males and females; that smokers had significantly lower levels of carotenoids; physically active people seem to favor the accumulation of carotenoids at higher levels than sedentary individuals; and that, in individuals with high baseline carotenoids, there is a more rapid cutaneous response to ozone exposure, while individuals with low baseline levels of SCS respond ineffectively to ozone.

Therefore, the present study reinforces the idea that pollution can affect skin homeostasis by depleting the presence of important protective molecules such as carotenoids, and their concentrations can be correlated with lifestyle habits such as smoking and physical activity. In addition, the data of this study suggest that carotenoid levels are a promising biomarker to establish skin health and pollution damage. Thanks to the non-invasive approach of Raman spectroscopy, this measurement could be part of our daily routine, allowing us to monitor outdoor stressor cutaneous damage. Finally, given the small population analyzed, further study with a more heterogenous and large population needs to be performed.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee: Comitato unico della provincia di FERRARA; Code: Eudract; Communication n.28 date of approval: 29 June 2017.

Informed Consent Statement: Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: Data are available upon request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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