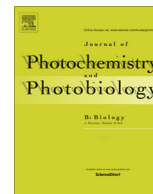




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# A dietary supplement improves facial photoaging and skin sebum, hydration and tonicity modulating serum fibronectin, neutrophil elastase 2, hyaluronic acid and carbonylated proteins

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## ABSTRACT

**Background and aims:** Excessive exposure to the sun can cause severe photoaging as early as the second decade of life resulting in a loss of physiological elastic fiber functions. We designed a first study to assess differences in facial skin pH, sebum, elasticity, hydration and tonicity and serum levels of fibronectin, elastin, neutrophil elastase 2, hyaluronic acid and carbonylated proteins between patients affected by facial photoaging and healthy controls. In a second study we tested the hypothesis that a dietary supplement would improve facial photoaging, also promoting changes in the above mentioned skin and serum parameters.

**Methods:** In the first study we enrolled 30 women [age:  $47.5 \pm 1.6$  years (mean  $\pm$  standard error of the mean)] affected by moderate facial photoaging ( $4 \text{ cm} \leq$  Visual Analogue Scale (VAS)  $< 7 \text{ cm}$ ) and 30 healthy women [age:  $45.9 \pm 1.6$  years (mean  $\pm$  standard error of the mean)]. In the second study we enrolled a cohort of 30 women [age:  $43.6 \pm 1.2$  years (mean  $\pm$  standard error of the mean)], affected by moderate ( $n = 22$ ) and severe ( $\text{VAS} \geq 7 \text{ cm}$ ;  $n = 8$ ) facial photoaging, who were randomized to receive a pharmaceutical formulation (VISCODERM® Pearls; IBSA FARMACEUTICI ITALIA Srl, Lodi, Italy) containing Pycnogenol®, collagen, coenzyme Q10, low-molecular-weight hyaluronic acid, chondroitin sulfate and glucosamine sulfate ( $n = 15$ ) or placebo ( $n = 15$ ). Dietary supplement and placebo were administered 2 times a day for 4 weeks. Facial photoaging was assessed by VAS in the first cohort of patients affected by facial photoaging and healthy controls and, at baseline and 2 weeks after the end of treatment, in the second cohort of patients who underwent treatment with VISCODERM® Pearls and placebo. Skin Tester was used to analyze differences in facial skin parameters between patients affected by facial photoaging and healthy controls. Skin Tester was also used to assess the effect of VISCODERM® Pearls on facial skin parameters and compared with placebo 2 weeks after the end of treatment. Serum levels of fibronectin, elastin, neutrophil elastase 2, hyaluronic acid and carbonylated proteins were measured by enzyme-linked immunosorbent assay in the first cohort of patients affected by facial photoaging and healthy controls and, at baseline and 2 weeks after the end of treatment, in the second cohort of patients who underwent treatment with VISCODERM® Pearls and placebo.

**Results:** VAS photoaging score was higher in patients affected by photoaging, if compared with healthy controls ( $p < 0.0001$ ). pH and sebum were increased in patients affected by photoaging, if compared with healthy controls (both  $p < 0.0001$ ), while elasticity, hydration and tonicity were decreased in patients affected by photoaging, if compared with healthy controls (all  $p < 0.0001$ ). Serum fibronectin and hyaluronic acid concentrations were lower in patients affected by photoaging, if compared with healthy controls (both  $p < 0.0001$ ). Serum neutrophil elastase 2, elastin and carbonylated protein concentrations were higher in patients affected by photoaging, if compared with healthy controls ( $p < 0.01$ ,  $p < 0.01$  and  $p < 0.0001$ , respectively). Dietary supplement administration resulted in an improvement in VAS photoaging score, if compared with placebo ( $p < 0.0001$ ), as observed 2 weeks after the end of treatment. Facial sebum, hydration and tonicity were increased in the active treatment group vs. placebo ( $p < 0.0001$ ,  $p < 0.0001$  and  $p < 0.05$ , respectively) 2 weeks after the end of treatment. Serum fibronectin and hyaluronic acid concentrations were increased in the dietary supplement group, if compared with placebo ( $p < 0.01$  and  $p < 0.001$ ) 2 weeks after the end of treatment, while no statistical difference in serum elastin concentration was observed between the two groups. Serum neutrophil elastase 2 and carbonylated protein concentrations

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were decreased in the dietary supplement group 2 weeks after the end of treatment, if compared with placebo ( $p < 0.001$  and  $p < 0.0001$ ).

**Conclusions:** We found significantly increased serum levels of neutrophil elastase 2, elastin and carbonylated proteins and decreased levels of hyaluronic acid and fibronectin in patients affected by facial photoaging, if compared with healthy controls. These findings coupled with a significant decrease in skin hydration, tonicity and elasticity and increased skin pH and sebum. Treatment with the dietary supplement VISCODERM® Pearls significantly improved VAS photoaging score and skin hydration, sebum and tonicity 2 weeks after the end of a 4-week treatment period in patients affected by moderate to severe facial photoaging. These findings coupled with a significant increase in serum fibronectin and hyaluronic acid and a decrease in serum carbonylated proteins and neutrophil elastase 2 in the active treatment group, if compared with placebo. Our findings suggest that VISCODERM® Pearls is effective for treatment of facial photoaging but further studies in larger cohorts of patients are required.

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## 1. Introduction

The main functions of the skin include temperature and pressure sensation and protection from external insults [1,2]. The skin can be divided into three layers, i.e. epidermis (characterized by a stratified squamous epithelium of proliferating basal and differentiated suprabasal keratinocytes), dermis (constituted by type I collagen fibrils, elastin fibers and glycosaminoglycans [GAGs]) and hypodermis (contains fibroblasts that play a critical role regulating extracellular matrix [ECM] organization, wound healing and interstitial fluid volume and pressure) [3–5]. Cutaneous aging is the result of chronological (innate) aging and sun-induced actinic damage that share the loss of normal elastic fiber functions as a key common feature [6]. Excessive exposure to the sun can cause severe photoaging as early as the second decade of life [7]. Ultraviolet (UV) radiation is classified according to the wavelength measured in the UVC, UVB and UVA ranges [8] and causes mutations by generating reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical [9]. Chronic UV exposure has also been implicated in skin photoaging [10]. For example UV irradiation increased expression of matrix metalloproteinases (MMP; degrade skin collagen and contribute to photoaging) in skin connective tissue and outer skin layers, if compared with non-irradiated skin layers [11]. Furthermore, in the same clinical study, the degradation of endogenous type I collagen fibrils was increased by 58% in irradiated skin, if compared with non-irradiated skin [11]. A decreased collagen production in dermal fibroblasts and a subsequent fragmentation and disarray of dermis collagen fibrils are also features of age-related phenotypic alterations in human skin [12,13]. The skin aging process can be targeted using instrumental treatments, topical creams/ointments and orally administered antioxidants that aim at scavenging both endogenous and exogenous free radicals [14]. Dietary formulations have been tested in patients presenting chronological aging and photoaging of the skin. For instance, a study, involving 53 female volunteers, studied the efficacy of a 5-week oral treatment with a dietary supplement containing N-acetyl-D-glucosamine, glucosamine sulfate, L-proline, manganese, copper, zinc, quercetin, and grape seed extract on skin aging [15]. The authors observed a significant reduction in the number of visible wrinkles and fine lines, while no changes in epidermal hydration occurred. Furthermore, a compound, containing hydrolyzed collagen type II, low-molecular-weight hyaluronic acid (HA) and chondroitin sulfate, increased hemoglobin, an indicator of microvascular blood circulation, 12 weeks post-treatment and collagen in the skin dermis at 6-week follow-up in patients presenting signs of innate aging and photoaging [16]. These changes coupled with a significant decrease in skin dryness/scaling, facial lines and wrinkles after a 12-week treatment period.

We designed a first study to assess differences in facial skin pH, sebum, elasticity, hydration and tonicity and serum levels of

fibronectin, elastin, neutrophil elastase 2, HA and carbonylated proteins between patients affected by facial photoaging and healthy controls. In a second study we tested the hypothesis that a dietary supplement containing Pycnogenol®, low-molecular-weight HA, collagen, glucosamine sulfate, chondroitin sulfate and coenzyme Q10, would improve facial photoaging modulating the above mentioned skin and serum parameters. We chose these serum parameters since they have been involved in the photoaging process as we will describe below.

### 1.1. Fibronectin

Fibronectin is a dimeric protein, composed of 250-kDa subunits [17], which exists in plasma and cellular forms and is a key component of the ECM [18]. The ECM is composed of fibrous structural proteins such as elastin and collagen, gel matrix, including proteoglycans and GAGs, and adhesive glycoproteins including fibronectin [19]. Serum elevation and dermal matrix deposition of fibronectin have been reported in patients affected by perforating disorders of the skin [20]. Regarding the effects of UV exposure of healthy human skin on fibronectin expression, conflicting results have been reported. For instance, in the experimental setting, Chen and colleagues reported that exposure to selective UVA irradiation did not change the expression of fibronectin in cultured human fibroblasts [21]. However, UVA and UVB irradiation of albino hairless mice produced a significant increase in fibronectin biosynthesis, as observed in skin cell cultures [22]. Similar results were reported by Schwartz and coworkers who showed that sunlamp irradiation (UVA and UVB) of hairless mouse skin resulted in an increase in fibronectin, as assessed by immunofluorescence [23]. In the same study, irradiated mouse skin contained 1.12 mg of extracted fibronectin per gram wet weight, if compared with 0.59 mg found in control skin.

### 1.2. Elastin

Elastin is an insoluble polymer of the monomeric soluble precursor tropoelastin and is the main component of elastic fibers in matrix tissue where it provides elastic recoil and resilience to a variety of connective tissues [24]. Solar radiation induces degenerative changes in the elastic fibers within the exposed skin [25] such as overproduction and basophilic degeneration up to destruction of the elastin meshwork in the dermis [26–28] and superficial vessels [29]. In addition, photoaged skin is characterized by an excessive deposition of abnormal elastic fibers [30].

### 1.3. Neutrophil elastase 2

Neutrophil elastase 2 is a major product of neutrophils and is strongly associated with solar elastosis in mice [31]. This potent proteolytic enzyme can degrade elastic and collagen fibers [32]

and its biological functions include involvement in migration of neutrophils by means of focal proteolysis, killing of microorganisms, and degradation/activation of various proteins including cytokines, chemokines and ECM proteins [33,34].

#### 1.4. Hyaluronic acid

HA is an ubiquitous linear polysaccharide assembled at the inner plasma membrane by HA synthases (HAS) [35,36]. HAS1 and HAS2 synthesize HA with a molecular mass of  $2 \times 10^5$  Da to approximately  $2 \times 10^6$  Da, whereas HAS3 synthesizes HA with a molecular mass of  $1 \times 10^5$ – $1 \times 10^6$  Da [37]. The synthesized HA activates Toll-like receptors 2 and 4 modulating inflammatory responses [38,39]. In the skin, HA plays a variety of important physiological roles in tissue hydration and mechanical protection due to its physicochemical properties such as strong hydration and viscoelasticity [40]. Moreover, previous *in vitro* and *in vivo* studies have shown a loss of HA from the dermis as a consequence of skin aging, thus suggesting a key role of HA in photoaging [41,42]. The effects of UVB on dermal HA have also been investigated, underlying that a chronic UVB exposure induces a progressive loss of HA from the upper dermis because of transcriptional down-regulation of all three HAS isoforms [35].

#### 1.5. Carbonylated proteins

Carbonylated proteins have been used as a measure of protein oxidation [43]. They may be formed either by oxidative cleavage of proteins or by direct oxidation of lysine, arginine, proline, and threonine residues [43]. A study showed that carbonylated proteins were increased within the upper dermis of chronically UV-exposed biopsies, if compared with intrinsically aged skin and young control skin, suggesting oxidative stress and oxidative protein damage in this area [43]. A 21-fold increase in carbonylated proteins in the upper part of the dermis has also been reported following chronic UVB irradiation, if compared with non-UVB exposed animals [44]. In a further study subcytotoxic UVA doses from 10 to 50 J per cm<sup>2</sup> induced a dose-dependent increase in carbonylated proteins in human fibroblasts [43].

## 2. Materials and methods

This study was performed at the Poliambulatorio del Secondo Parere (Modena, Italy) in accordance with the Declaration of Helsinki and approved by the local Institutional Review Board. The patients signed the informed consent and underwent a blood test to assess liver and kidney function before and after the dietary supplement intervention to monitor the treatment safety profile.

#### 2.1. Patients

In the first study we enrolled 30 women [age:  $47.5 \pm 1.6$  years (mean  $\pm$  standard error of the mean [SEM])] affected by moderate facial photoaging [4 cm  $\leq$  Visual Analogue Scale (VAS) < 7 cm] and 30 healthy women [age:  $45.9 \pm 1.6$  years (mean  $\pm$  SEM)] to study differences in selected serum (fibronectin, elastin, neutrophil elastase 2, HA and carbonylated proteins) and skin (pH, sebum, elasticity, hydration and tonicity) parameters. In the second study we enrolled a second cohort of 30 women [age:  $43.6 \pm 1.2$  years (mean  $\pm$  SEM)] affected by moderate ( $n = 22$ ) and severe (VAS  $\geq 7$  cm;  $n = 8$ ) facial photoaging that were randomized to receive a pharmaceutical formulation, VISCODERM® Pearls (IBSA FARMACEUTICI ITALIA Srl, Lodi, Italy;  $n = 15$ ) or placebo ( $n = 15$ ). All patients had Fitzpatrick skin type 2 or 3. Inclusion criteria were moderate facial photoaging for the first cohort of patients and

moderate to severe facial photoaging for the second cohort of patients, as determined by VAS. Exclusion criteria were as follows: skin allergies, face dermatitis, pre-cancerous facial skin lesions due to prolonged UV exposure and skin or general symptoms of intolerance/allergy to the ingredients contained in the dietary compound used in this study.

#### 2.2. Pharmacological treatment

The second cohort of 30 patients was divided into 2 groups and randomized to receive (1) VISCODERM® Pearls containing Pycnogenol® (15 mg), collagen (124 mg), chondroitin sulfate (40 mg), glucosamine sulfate (not less than 3%), low-molecular-weight HA, (20 mg) and coenzyme Q10 (10 mg) 2 times a day for 4 weeks or (2) a placebo formulation containing starch (200 mg) 2 times a day for 4 weeks. Both placebo and active treatment groups received a standardized diet (1800 kcal per day including 20% proteins, 50% carbohydrates and 30% fats) in order to allow comparison between groups without any diet-related bias.

Collagen was introduced into the dietary compound since experimental evidence from mouse models has shown that: (1) it is protective against UV-induced skin damage [45]; (2) damage to collagen and elastic fibers leads to wrinkling and skin laxity suggesting that collagen plays a key role in the development of these conditions [15]. Furthermore, the ingestion of hydrolyzed collagen resulted in detection of hydroxyproline-containing peptides in human blood [46]; these peptides have demonstrated to stimulate human dermal fibroblasts to synthesize HA *in vitro* [46]. HA was also included in the dietary formulation due to its structural function in the skin where it can bind water supporting volume expansion and turgidity, metabolite and nutrient diffusion and elasticity [35]. Furthermore, a decreased amount of HA has been ascribed to innate aging and photoaging processes [47,48]. Aging-related decrease in GAGs including HA leads to wrinkling and altered elasticity [47]. Taken together, this evidence supports a role of HA in skin aging and photoaging. We included chondroitin sulfate since it plays a key role in collagen and elastic fiber formation [49]. The reason for glucosamine sulfate inclusion in the dietary compound is previous evidence showing that, when combined with other compounds, improves cutaneous aging-induced wrinkles [15]. Furthermore, N-acetylglucosamine inhibited UVB-induced collagenolytic MMP production through different pathways in immortalized human skin fibroblasts [50]. Exogenous antioxidants have been extensively used to target pathology-related oxidative stress in different body systems, as previously reviewed [51,52]. Specifically, topical Pycnogenol® and oral coenzyme Q10 have shown promising results in the treatment of photoaging-induced oxidative damage at the level of the skin as reviewed by Berson [53]. Furthermore, Pycnogenol® improved skin elasticity and hydration in women presenting dry skin [54]. Coenzyme Q10 increased fibroblast proliferation, augmented expression of type IV collagen and reduced UV radiation-induced MMP-1 level in embryonic and adult cells (type IV collagen and MMP-1 have been implicated in photoaging [55]) [56].

Furthermore, treatment with coenzyme Q10 increased elastin gene expression in cultured fibroblasts and decreased UV radiation-induced interleukin-1 alpha (IL-1 $\alpha$ ) production in HaCaT cells [56]. This evidence explains the inclusion of Pycnogenol® and coenzyme Q10 into the dietary formulation object of our study.

#### 2.3. Assessment of photoaging

Photoaging was quantified by VAS by an esthetic surgeon in the first cohort of patients and, at baseline and 2 weeks after the end of treatment, in the second cohort of patients.

#### 2.4. Blood collection and measurement of serum parameters

Blood samples were collected from the patients' cubital veins at baseline and 2 weeks after the end of treatment and were transferred into a heparin-coated tube (Becton Dickinson Italia, Buccinasco, Milan). The serum was separated by low-speed centrifugation at 1000 rpm (ultra select LW-U8S-2, LW Scientific Inc., Atlanta, USA) for 10 min and then stored at  $-20^{\circ}\text{C}$ . The serum levels of elastin, neutrophil elastase 2, fibronectin, HA and carbonylated proteins were detected using enzyme-linked immunosorbent assay (ELISA) (SEB337Hu and SEA181Hu, Cloud-Clone Corp., Houston, TX, USA; ab108848, abcam, Cambridge, UK; TE1017-2, TECOmedical AG, Sissach, Switzerland; K7822, Immundiagnostik AG, Bensheim, Germany) according to the manufacturers' instructions. The ELISA for elastin detects amino acids Gly392 ~ Ala645 and therefore can detect isoforms 1, 2 and 6 as described by the manufacturer. The ELISA for HA detects mainly high molecular weight HA and very high molecular weight HA, as reported by the manufacturer. However, very low molecular weight HA and medium molecular weight HA are also detected. The ELISA for fibronectin recognises recombinant, natural and fragmented forms of fibronectin (tropoelastin is not detected). A microplate reader (Spectrostar Nano, EuroClone S.p.a., Milano, Italy) was used for measurement of elastin, elastase 2, fibronectin, HA and carbonylated protein concentrations.

#### 2.5. Skin parameter clinical evaluation

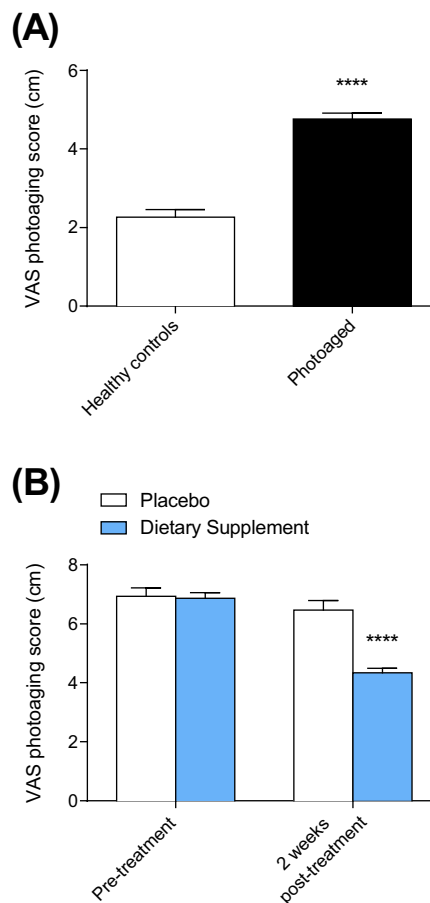
Skin Tester [manufactured by Selenia Italia (Pisa, Italy) and distributed by Dermal Medical Division (Bologna, Italy)] was used to quantify facial pH, hydration, sebum, elasticity and tonicity in the first cohort of patients affected by facial photoaging and healthy controls and, at baseline and 2 weeks after the end of treatment, in the second cohort of patients who underwent treatment with VISCODERM® Pearls and placebo. This instrument consists in an ultrasound emitted beam that is reflected by the dermal tissues according to their stromal density and vascular tone. Furthermore, impedance variation, related to intracellular and interstitial water content and vascular network dynamics (evaluated by photoplethysmography and reflectometric method), are all assessed by the same diagnostic device. We have previously used Skin Tester to successfully determine the efficacy of HA-based cosmetic procedures [57].

#### 2.6. Statistical analysis

All data are presented as the means  $\pm$  SEM. A two-way Analysis of Variance (ANOVA) with Sidak's multiple comparisons test was used to analyze differences in skin parameters, serum fibronectin, elastin, neutrophil elastase 2, HA and carbonylated protein concentrations and VAS photoaging score pre- and post-treatment between dietary supplement group and placebo. An unpaired 2-sample Student's *t*-test was used to compare differences in VAS photoaging score, serum fibronectin, elastin, neutrophil elastase 2, HA and carbonylated protein concentrations and skin parameters between healthy and photoaged patients. All statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).  $p < 0.05$  was considered significant.

### 3. Results

At the end of the 4-week dietary supplement treatment period, blood tests for liver and kidney functions were within normal range.



**Fig. 1.** VAS photoaging score is significantly increased in patients affected by photoaging, if compared with healthy controls (A). Dietary supplement significantly improves VAS photoaging score 2 weeks after the end of treatment, if compared with placebo (B). \*\*\*\* $p < 0.0001$ .

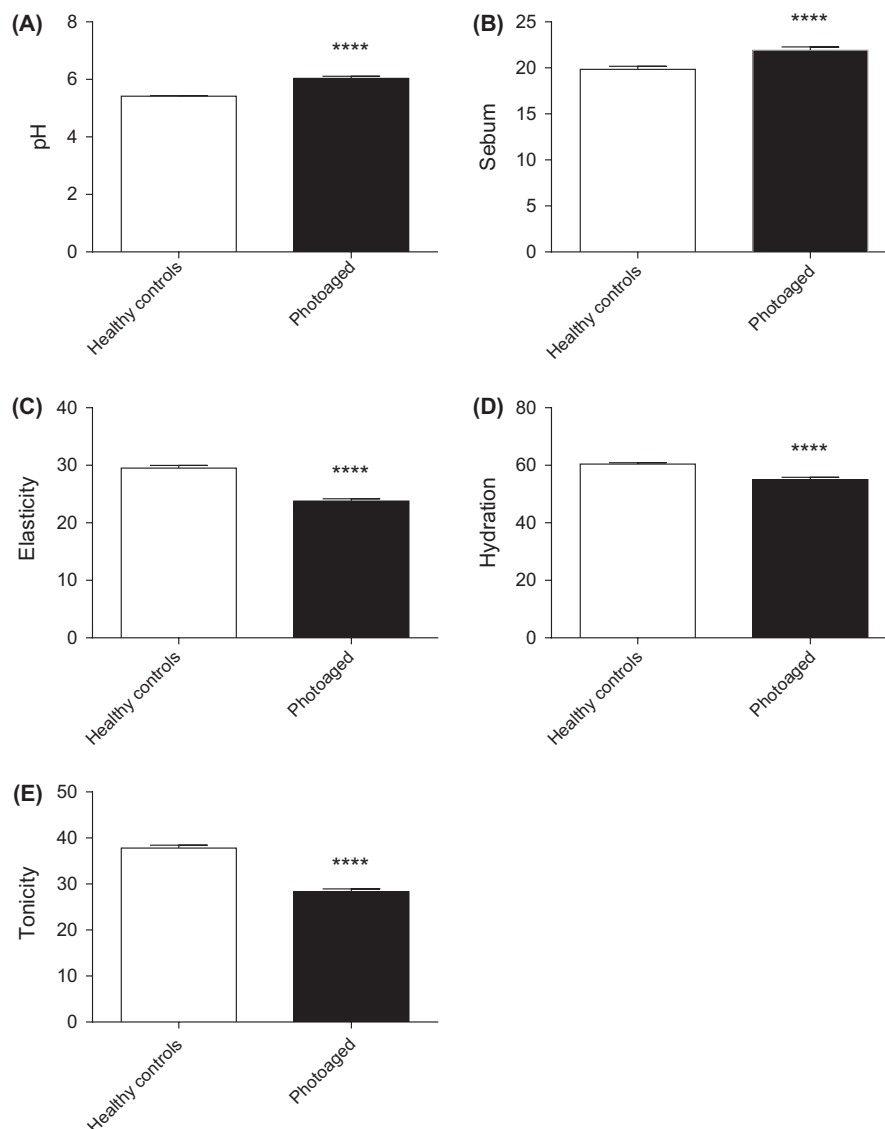
#### 3.1. Efficacy of treatment on photoaging

VAS photoaging score was higher in the patients affected by photoaging ( $4.7 \pm 0.1$  cm), if compared with healthy controls ( $2.2 \pm 0.1$  cm;  $p < 0.0001$ ; Fig. 1A). Two weeks after the end of pharmacological treatment, an improvement in VAS photoaging score was observed in patients treated with the dietary supplement ( $4.3 \pm 0.1$  cm), if compared with placebo ( $6.4 \pm 0.3$  cm;  $p < 0.0001$ ; Fig. 1B).

#### 3.2. Skin parameters

Dehydration, atrophy and loss of elasticity characterize the aged skin [58]. In the present study pH and sebum were increased in patients affected by photoaging, if compared with healthy controls (both  $p < 0.0001$ ; Fig. 2A and B). Elasticity, hydration and tonicity were decreased in patients affected by photoaging, if compared with healthy controls (all  $p < 0.0001$ ; Fig. 2C–E). Previous studies in our clinic used Skin Tester to determine changes in skin parameters following skin rejuvenation procedures [57]. In the present study no difference in facial skin pH (Fig. 3A) and elasticity (Fig. 3C) was observed between placebo and dietary supplement groups 2 weeks after the end of treatment. Facial sebum, hydration and tonicity were increased in the active treatment group vs. placebo 2 weeks after the end of treatment (Fig. 3B, D and E;  $p < 0.0001$ ,  $p < 0.0001$  and  $p < 0.05$ , respectively).





**Fig. 2.** Skin pH (A) and sebum (B) are significantly increased in patients affected by photoaging, if compared with healthy controls. Skin elasticity (C), hydration (D) and tonicity (E) are significantly decreased in patients affected by photoaging, if compared with healthy controls. \*\*\*\* $p < 0.0001$ .

### 3.3. Fibronectin

Fibronectin fragmentation is involved in the skin-related modifications occurring after UV exposure [59]. We found that serum fibronectin concentration was lower in patients affected by photoaging ( $9.07 \pm 0.5 \mu\text{g/ml}$ ), if compared with healthy controls ( $23.9 \pm 1.6 \mu\text{g/ml}$ ;  $p < 0.0001$ ; Fig. 4A). Dietary supplement administration resulted in an increase in serum fibronectin ( $12.07 \pm 0.8 \mu\text{g/ml}$ ) 2 weeks after the end of treatment, if compared with placebo ( $9.3 \pm 0.7 \mu\text{g/ml}$ ;  $p < 0.01$ ; Fig. 5A).

### 3.4. Neutrophil elastase 2

Several studies have highlighted the role of neutrophil elastase 2 in the ECM damage due to UV exposure [31,60,61]. We found that serum elastase 2 concentration was higher in patients affected by photoaging ( $13.6 \pm 0.7 \text{ pg/ml}$ ), if compared with healthy controls ( $10.9 \pm 0.5 \text{ pg/ml}$ ;  $p < 0.01$ ; Fig. 4B). Conversely, serum neutrophil elastase 2 concentration was significantly decreased in patients treated with the dietary supplement ( $9.7 \pm 1.9 \text{ pg/ml}$ ), if

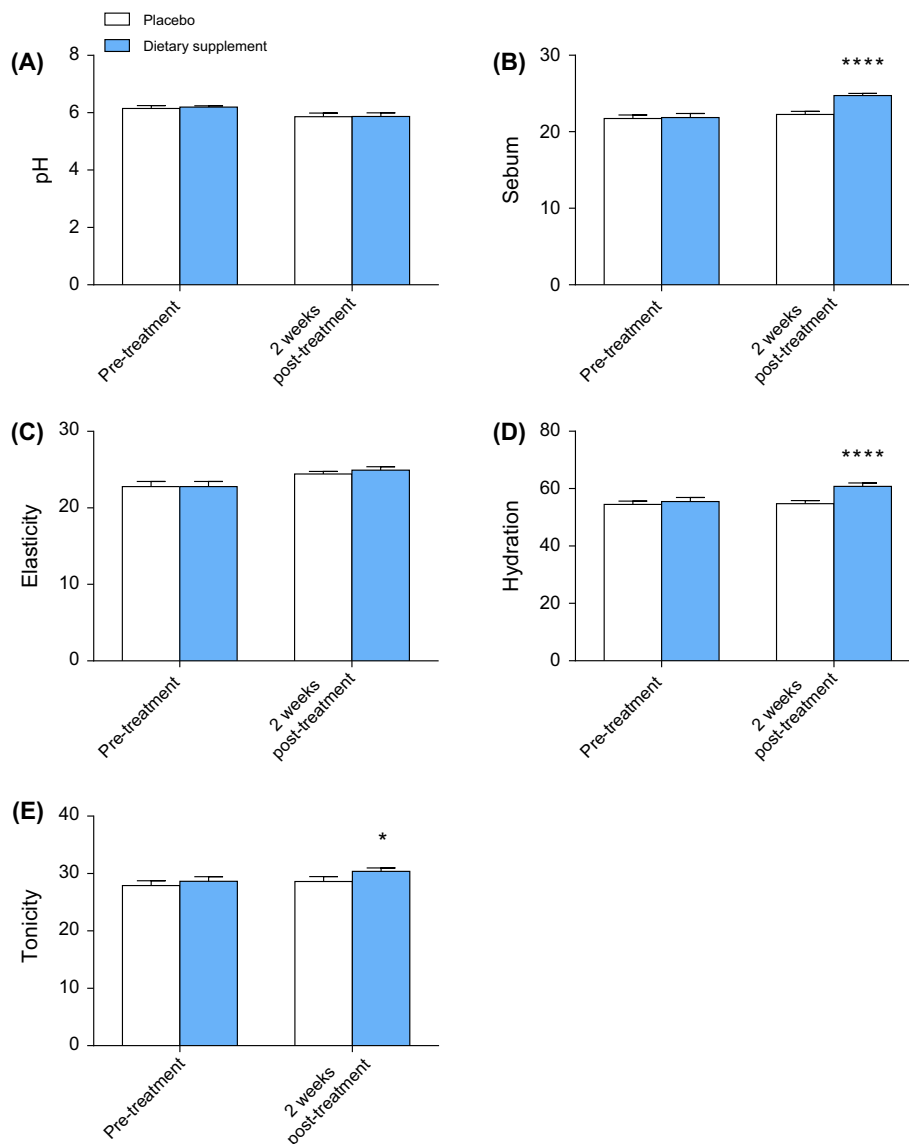
compared with subjects receiving placebo ( $13.3 \pm 1.5 \text{ pg/ml}$ ;  $p < 0.001$ ; Fig. 5B) 2 weeks after the end of treatment.

### 3.5. Elastin

Extracellular space abnormal accumulation along with intracellular degradation of elastin have been observed in photoaged skin [62]. We found that serum elastin concentration was higher in patients affected by photoaging ( $10.6 \pm 0.7 \text{ ng/ml}$ ), if compared with healthy controls ( $8.2 \pm 0.4 \text{ ng/ml}$ ;  $p < 0.01$ ; Fig. 4C). No difference in serum elastin concentration was observed between the dietary supplement ( $9.7 \pm 1.9 \text{ ng/ml}$ ) and placebo groups ( $12.5 \pm 0.9 \text{ ng/ml}$ ; Fig. 5C) 2 weeks after the end of treatment.

### 3.6. Hyaluronic acid

HA degradation has been widely used as a marker of photoaging [63]. In our study, we found that serum HA concentration was lower in patients affected by photoaging ( $19.6 \pm 1.1 \text{ ng/ml}$ ), if compared with healthy controls ( $37.1 \pm 1.8 \text{ ng/ml}$ ;  $p < 0.0001$ ; Fig. 4D). Dietary supplement administration resulted in an increase in serum HA concentration ( $33.2 \pm 3.2 \text{ ng/ml}$ ), if compared with



**Fig. 3.** No difference in skin pH (A) and elasticity (C) is observed between the dietary supplement and placebo groups. Sebum (B), hydration (D) and tonicity (E) are increased in the dietary supplement group, if compared with placebo. \* $p < 0.05$  and \*\*\*\* $p < 0.0001$ .

placebo ( $19.9 \pm 1.6$  ng/ml;  $p < 0.001$ ; Fig. 5D), 2 weeks after the end of treatment.

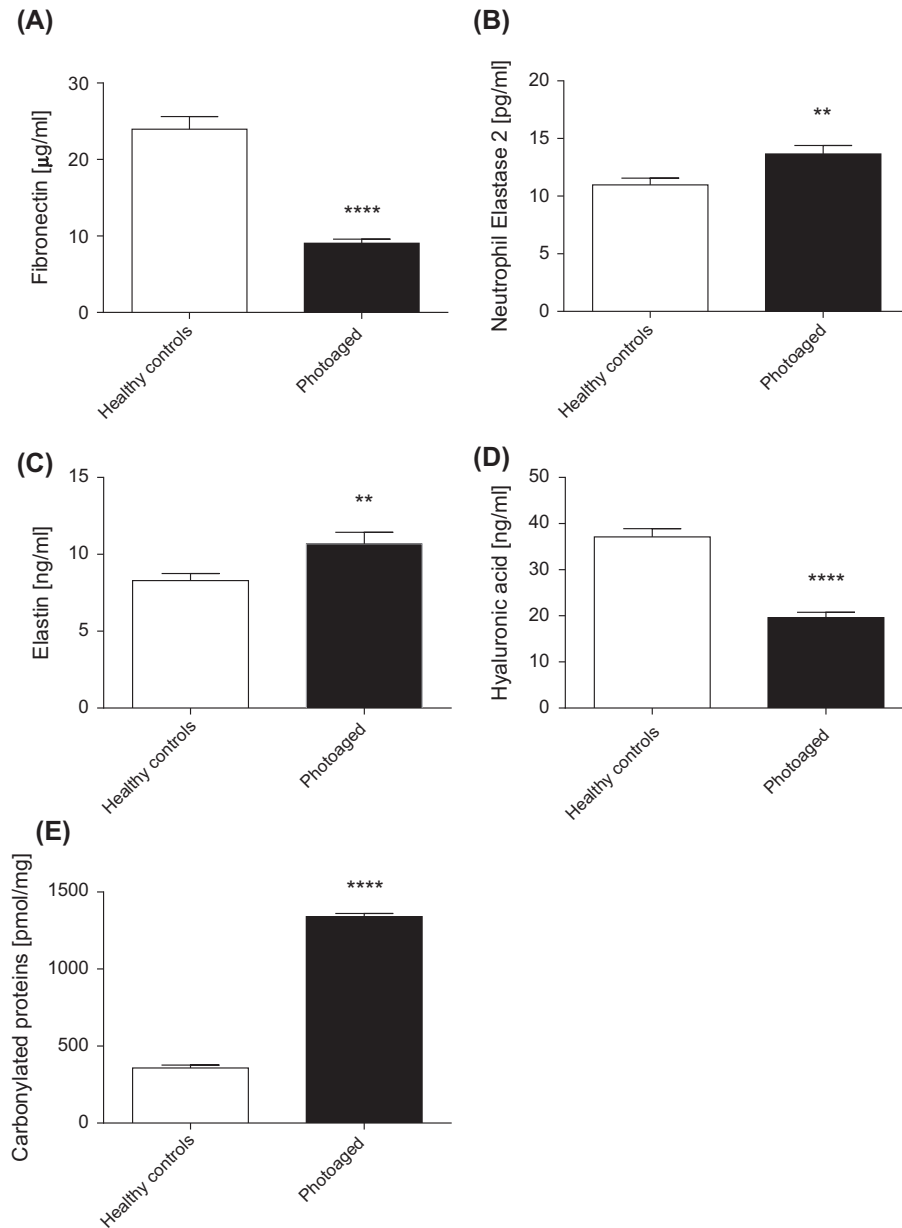
### 3.7. Carbonylated proteins

Carbonylated proteins are a marker of UV-induced oxidative damage [64]. Accordingly, we found that serum carbonylated proteins were higher in patients affected by photoaging ( $1340 \pm 20.2$  pmol/mg), if compared with healthy controls ( $357.5 \pm 19.3$  pmol/mg;  $p < 0.0001$ ; Fig. 4E). Dietary supplement administration resulted in a decrease in serum carbonylated proteins ( $358.4 \pm 25.5$  pmol/mg), if compared with placebo ( $978.3 \pm 32.2$  pmol/mg;  $p < 0.0001$ ; Fig. 5E), 2 weeks after the end of treatment.

## 4. Discussion and conclusions

We found significantly increased serum levels of neutrophil elastase 2, elastin and carbonylated proteins and decreased levels of HA and fibronectin in subjects affected by facial photoaging, if

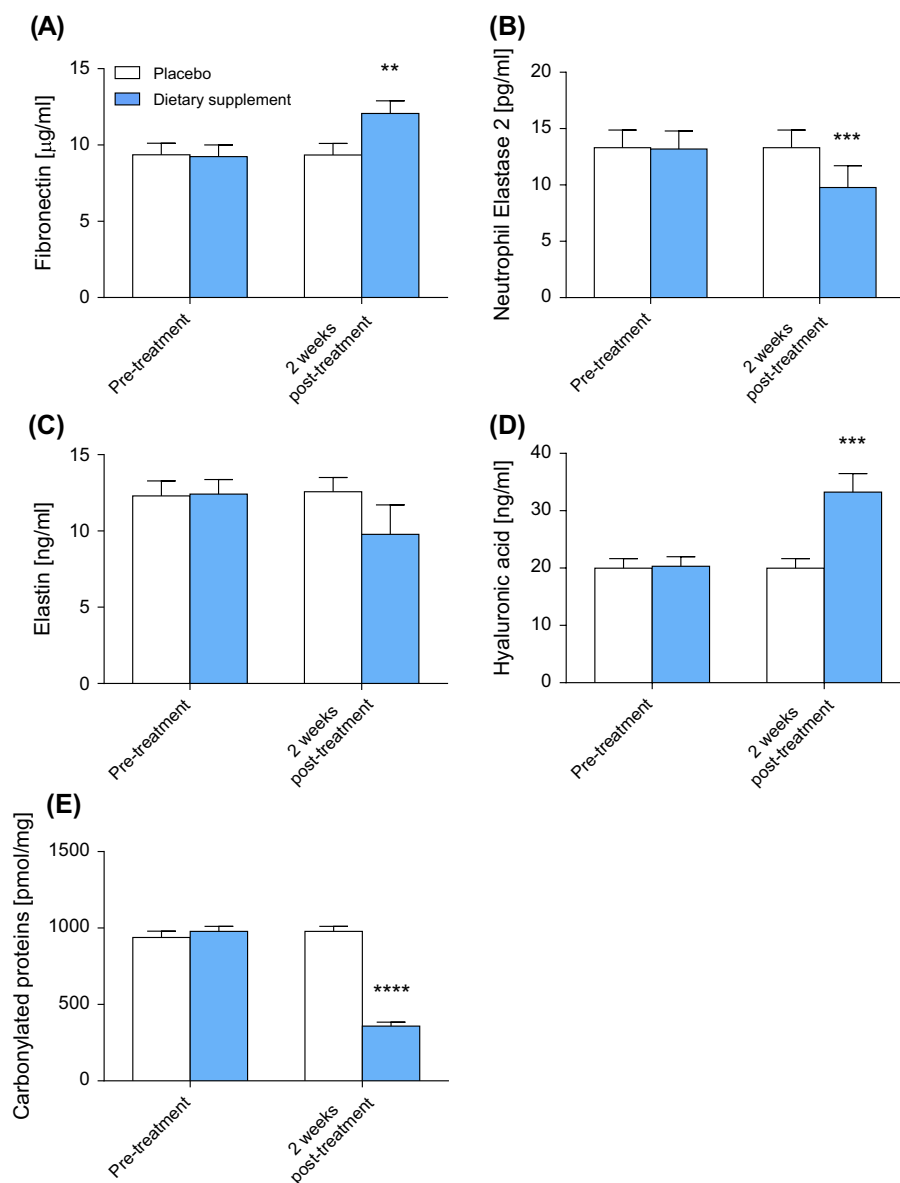
compared with healthy controls. These findings coupled with a significant decrease in skin hydration, tonicity and elasticity and increased skin pH and sebum. In our study, the changes observed in skin parameters are in agreement with the clinical symptoms of photoaged skin including loss of hydration, erythema, fine and coarse wrinkling, roughness, dryness, thickening, laxity, telangiectasias and loss of tensile strength [65]. The loss in hydration is due to the GAG increase which is observed in photoaged skin while, on the other hand, GAGs are decreased in innate aging [66]. In photoaged skin, GAGs are deposited on the abnormal elastotic material rather than in the papillary dermis becoming unavailable as a source of hydration [67]. Our study, reporting an increase in sebum in photoaged facial skin, is in agreement with an experimental study in hairless mice, a model of photoaging, showing that UV radiation causes marked hyperplasia of the sebaceous glands [68]. Specifically, UVA has been implicated in damaging the sebaceous glands as it can penetrate deeper into the dermis and can reach the sebaceous gland [69]. Skin pH is a key factor in maintaining the function and integrity of the stratum corneum, the body's first line of defense against environmental exposures such as solar UV radiation [70,71]. Therefore, our findings showing an increase



**Fig. 4.** Serum fibronectin (A) and hyaluronic acid (D) are significantly decreased in patients affected by facial photoaging, if compared with healthy controls. Serum neutrophil elastase 2 (B), elastin (C) and carbonylated proteins (E) are significantly increased in patients affected by photoaging, if compared with healthy controls. \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$ .

in skin pH, are in line with evidence showing that UV radiation compromises the barrier function of the skin affecting the stratum corneum intercellular components such as intercellular lipids and corneodesmosomes [71]. At the best of our knowledge we are the first to find a decrease in serum fibronectin in patients affected by photoaging, if compared with healthy controls. However, a previous study showed that plasma fibronectin increases with age [72]. A similar trend was observed in a study using skin explant cultures from hairless mice of increasing age showing that fibronectin biosynthesis increased progressively with age [73]. Therefore, we can argue that different mechanisms of fibronectin regulation are at play in aging and photoaging. Our findings showing a significant increase in serum neutrophil elastase 2 in patients affected by photoaging, if compared with healthy controls, are also novel. However, a study performed on fibroblast-like cells from specimens of sun-exposed areas showed a positive reaction for neutrophil elastase 2 [74]. A similar increase in elastase enzymatic

activity was observed in skin extracts from hairless mice following chronic UV irradiation [59]. Furthermore, neutrophil elastase 2 expression and neutrophil influx were investigated by Rijken et al. on snap-frozen punch biopsies of healthy volunteers exposed to solar-simulated radiation [75]. In Rijken's study, immunohistochemical staining showed that neutrophils were the major source of MMP-1, MMP-8 and MMP-9. Although we are the first to show an increase in serum elastin in patients affected by facial photoaging, Johnston and colleagues found an increase in elastin content in UVA- and UVB-exposed hairless mouse skin [76]. A dose-dependent increase in tropoelastin (the soluble precursor of elastin [77]) expression was also observed following sun exposure in photodamaged human skin fibroblasts [78]. To the best of our knowledge we are the first to investigate serum HA levels in patients affected by photoaging and we found a significant decrease in HA concentration in photoaged subjects, if compared with controls. However, Tzellos and coworkers reported elevated HA levels



**Fig. 5.** No difference in serum elastin concentration is observed between the dietary supplement and placebo groups (C). Serum fibronectin (A) and hyaluronic acid (D) are significantly increased while neutrophil elastase 2 (B) and carbonylated proteins (E) are significantly decreased in patients treated with the dietary supplement, if compared with placebo. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ .

in photoexposed human skin biopsies from facial regions, if compared with photoprotected skin from the area behind the ear lobe [63]. Our study also shows a significant increase in serum carbonylated proteins in patients affected by photoaging, if compared with healthy controls. Carbonylated proteins have been previously used as biomarker for oxidative stress or damage [79]. Sander et al. reported an accumulation of oxidatively modified proteins, specifically within the upper dermis of photoaged skin, upon acute UV exposure of healthy subjects, showing a correlation between photodamage and protein oxidation [43]. Therefore, a significant decrease in serum carbonylated proteins in patients affected by photoaging supports the hypothesis of an overall reduced oxidation state.

In the present study, administration of a dietary supplement containing Pycnogenol<sup>®</sup>, low-molecular-weight HA, collagen, glucosamine sulfate, chondroitin sulfate and coenzyme Q10 significantly improved facial photoaging, as assessed 2 weeks after the end of a 4-week treatment period. This finding coupled with a significant increase in sebum, hydration and tonicity, if compared

with placebo. The improvement in facial photoaging also coupled with an increase in serum fibronectin and HA and a decrease in serum carbonylated proteins and neutrophil elastase 2. Our findings suggest a systemic modulation of these parameters that may represent biomarkers of photoaging pathology and could be used to monitor progression or improvement in this condition. The results from this study are in agreement with a previous investigation where a dietary formulation (BioCell Collagen<sup>®</sup>) administered for 12 weeks improved skin dryness/scaling and global lines/wrinkles with a significant increase in hemoglobin and collagen in skin dermis at 6 weeks and hemoglobin at the end of the study [16]. In summary, our dietary compound shows a synergistic efficacy of its individual ingredients in improving facial photoaging 2 weeks after the end of a 4-week treatment period. Pycnogenol<sup>®</sup>, HA, collagen, glucosamine sulfate, chondroitin sulfate and coenzyme Q10, which all possess a rationale of efficacy in modulating the ECM, can be put together in a dietary compound that produces an improvement in skin photoaging also modulating serum HA, carbonylated proteins, fibronectin and neutrophil elastase 2 levels. The clinical meaning of



these parameters and their involvement in the photoaging pathophysiology at the systemic level warrant further investigation. Future studies will also address the long-term effects of the formulation used in this investigation in patients affected by photoaging. A limitation of our study is that the ELISAs we used do not allow to discriminate among different forms of some of the analytes that have been the object of our investigation.

### Conflict of interest statement

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this manuscript. This study was performed according to the Declaration of Helsinki and local internal review board rules.

### Informed consent

Written informed consent was obtained from the patients. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

### Statement of authorship

The authors hereby certify that all work contained in this article is original. The authors claim full responsibility for the contents of the article.

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