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Original Article

# Clinical, biometric and structural evaluation of the long-term effects of a topical treatment with ascorbic acid and madecassoside in photoaged human skin

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**Abstract:** Skin ageing is a complex process determined by the genetic endowment of individual and environmental factors, such as sun exposure. The effects of skin ageing are mostly encountered in the superficial dermis and in the epidermis. We have previously demonstrated *in vivo* the beneficial effect of a topically applied formula of 5% vitamin C in the treatment of skin ageing. Another active compound, madecassoside extracted from *Centella asiatica*, known to induce collagen expression and/or to modulate inflammatory mediators, might thus prevent and correct some signs of ageing. A randomized double-blind study was carried out on photoaged skin of 20 female volunteers to investigate the effects of topically applied 5% vitamin C and 0.1% madecassoside on the clinical, biophysical and structural skin properties. After 6 months of treatment, we observed a significant improvement of the clinical score for deep and superficial wrinkles, suppleness, firmness, roughness and skin hydration. These results were corroborated by measurements of skin elasticity and semi-quantitative histological assessment of the elastic fibre network in the papillary dermis. Two-thirds of the subjects showed an improvement. The re-appearance of a normally structured elastic fibre network was observed. Our results revealed a functional and structural remodelling of chronically sun-damaged skin.

**Key words:** elastic fibres – madecassoside – photoageing – skin ageing – vitamin C

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## Introduction

Cutaneous ageing depends on intrinsic factors (chronologic ageing) and on deleterious influences from the environment (mainly ultraviolet radiation). The resulting effects such as skin laxity, sagging and wrinkle formation are clinically obvious and concern mainly the superficial dermis. The epidermis, largely exposed to environmental assaults, shows in the long run signs of atrophy, flattening of the dermal-epidermal junction, pigmentary changes, irregular desquamation and precancerous or neoplastic lesions (1). While 70% of the short ultraviolet waves (UVB) are already absorbed in the horny layer loosing most of their energy, long ultraviolet light (UVA) easily penetrates into the papillary and superficial reticular dermis (Fig. 1) (2). The elastin and collagen fibre networks appear to be the primary targets of UVA exposure. The density of the collagen I fibres is largely reduced in the actinically altered superficial dermis and the collagen bundles show less anisotropic orientation (3). Elastic fibres of the oxytalan type, perpendicular and attached to the dermal-epidermal junction, progressively disappear from the papillary dermis (4). Elaunin arches, parallel to the skin surface, to which oxytalan fibres are attached at the limit between the papillary and reticular dermis, become fragmented. Finally, elastotic material derived from degraded elastic tissue accumulates in the upper reticular dermis (4–6). During ageing, ultrastructural changes in the elastic fibres seem to progress from localized deposition of osmiophilic materials to the substitution of the great majority of the amorphous elastin with interwoven filaments, negative for elastin specific antibodies (7). Amorphous components of the extracellular matrix decrease in aged skin resulting in the presence of hollow spaces between the compacted fibre bundles (8). At the same time, collagen production in the super-

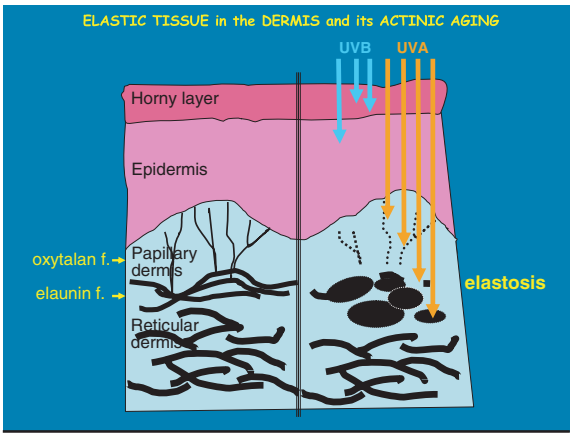


**Figure 1.** Schematic visualization of the impact of ultraviolet radiation on human skin. Long wavelength UVA rays (320–400 nm) are able to penetrate into the papillary dermis with enough energy to cause fibrillar matrix damage. The deleterious changes present as elastic fibre fragmentation and elastosis combined with rarefaction and disorganization of collagen fibres in the papillary and superficial reticular dermis. The shorter UVB radiation (290–320 nm) is largely adsorbed in the stratum corneum and viable epidermal layers, where it exerts its action.

ficial dermis slows down and the resulting changes are reminiscent of another senescence process, osteoporosis (9). The above described changes are partly due to a decreased fibroblast activity in chronologically aged skin (10). Senescence of cells within the epidermal and dermal compartments is regulated by the rate of chromosomal telomere decay and affected by locally generated reactive oxygen species (11). Thus, prevention and correction of the skin ageing process require protection of the existing functional structures and of the proliferative and metabolic potential of skin cells. We have previously demonstrated the beneficial effects of topically applied ascorbic acid on the synthesis of essential structural dermal elements, i.e. collagens I and III and elastic fibres, leading to a partial restoration of the mechanical and surface properties in chronically and actinically aged human skin (12,13). In the present study, vitamin C was combined with madecassoside, a compound capable of stimulating collagen I synthesis (14–17) and of generating a synergistic anti-ageing effect.

## Materials and methods

### Volunteers and treatment

A randomized double-blind study versus a plain moisturizing cream was carried out on 20 healthy postmenopausal female volunteers (45–60 years old; mean ± SD = 51.1 ± 4.3 years) with actinically aged facial, neck and forearm skin. The control cream (Toleriane®; La Roche-Posay Laboratoire Pharmaceutique, Asnières, France) contained no specific active compounds except for 6% glycerine. Informed consent was obtained from all volunteers, in agreement with the Declaration of Helsinki, and the study was approved by the Ethics Committee of Saint-Jacques University Hospital in Besançon, France. Subjects with a known allergy to any of the creams' ingredients, or those having used any other 'anti-ageing' cream on the region of interest (ROI) during the preceding month, were not included. No topical application of any other product onto the ROI was permitted. The other exclusion criteria were acute or chronic illness likely to necessitate a treatment with corticoids, topical vitamin A acid or other alpha-hydroxy-acid treatment during the month before the beginning of the study and disorders resulting from excessive alcohol or toxic substance consumption. The tested cream contained 5% stabilized vitamin C and 0.1% madecassoside (Redermic®; La Roche-Posay Laboratoire Pharmaceutique).

A fingertip unit of the tested cream was applied twice daily for 6 months to the face, as well as the assigned half of the neck and upper chest, and one of the arms of each volunteer whereas the other half of the neck and the other arm received the control cream. Each patient received a randomized pair of strictly identical tubes, colour coded and labelled right or left, as well as a third tube for the application on the face. Among these 20 patients, 14 accepted to have biopsies on both arms at the beginning as well as at the end of the trial (before and after 6 months of treatment).

### Noninvasive evaluations

Clinical examination, self-evaluation by volunteers and noninvasive skin assessments were performed before the treatment, then after 1.5, 3, 4.5 and 6 months.

Scores by the investigator were as follows. Hydration was graded: 0, normal; 2, dry or 4, very dry. Roughness and laxity were graded: 0, null; 2, mild; 4, moderate; 6, pronounced. Suppleness was graded: 0, pronounced; 2, moderate; 4, mild; 6, absent. Fine wrinkles and coarse wrinkles were graded from 0 to 14. Radiance was graded: 1, high; 2, normal; 3, lustreless; 4, dull. Brown spots were graded: 0, absent; 2, visible; 4, pronounced. Skin homogeneity was graded as: 0, non-homogeneous; 2, homogeneous. The calculated global clinical score represented the sum of the eight previously mentioned items. The maximal score, corresponding to the most pronounced alterations was therefore 50. Additionally, at the beginning of the trial and during every visit, the investigator also assessed overall skin sensitivity to the treatment at each ROI; desquamation, erythema, stinging or burning sensations were graded: 0, absent; 1, mild; 2, moderate or 3, severe. The analysed population was the population with 'intention-to-treat'. Quantitative variables were described by the mean, the standard error of the mean, the median, the minimum and the maximum. For the analysis of the evolution of the global score on the neckline during the trial the Friedman's test completed by the Dunn's test on the differ-

 

ence were performed (score assessed on the side treated with active compounds minus score from the control side). For the analysis of both the investigator's and the volunteer's judgements, the percentages were compared between each group. Dunnett's test was used to assess the additional benefit obtained with the active compounds over the control cream ($P < 0.05$ was considered significant).

The volunteers were asked to score from 0 (unsatisfactory) to 10 (very satisfactory), on an analogical scale, the state and evolution of each of the following items: hydration, fine wrinkles, coarse wrinkles, firmness, radiance, withering, tonicity, suppleness, pigmented spots, roughness, comfort, smoothness, imperfections, smoothing (=sleekness), dryness, erythema. The results were recorded for each coded and randomly assigned ROI.

Biometrological measurements were performed at each visit on each ROI (face and both sides of the neckline). The hydration index was assessed using measurements of impedance (Corneometer CM825; Courage & Khazaka, Koeln, Germany), the skin elasticity by cutometry (Cutometer; Courage & Khazaka), and the skin relief was studied by fringe projections *in vivo* (LIBC prototype; 18). In this latter method, a network of light fringes of various widths was projected on the test area and became distorted by the relief. Four different data acquisition processes were performed and the 3D profile of the area was reconstituted by the computer by calculating the height of each spot on the examined surface. The parameters of wrinkle shape, i.e. depth and volume, were extracted from these images for the analysis of the 'crow's feet' of the face as well as the parameters of micro relief (roughness) on the neck.

The treatment-induced improvement was presented as percent change (mean ± SD), i.e. increase or decrease, depending on the evaluated item, when compared with the initial value. An analysis of variance (ANOVA) on repeated data completed by a Student–Newman–Keuls test (normal distribution of parameters) or a Friedman test completed by a Dunn's test (abnormal distribution of parameters) were applied to analyse the evolution of the studied parameters over the chosen time period. Statistical significance was considered when $P < 0.05$.

## Histology, histochemistry and ultrastructure

Skin biopsies were taken from both arms, under local anaesthesia, before and after 6 months of treatment in 14 volunteers. The biopsies were processed for standard electron microscopy (EM) and light microscopy (LM) studies aiming at evaluation of epidermal differentiation and papillary dermis structure. For standard histology and immunohistochemistry, the skin fragments were fixed in 10% formalin (Baker's fixative) and embedded in paraffin. Five micrometre sections were dewaxed, rehydrated and stained with haematoxylin-phloxin-safran (HPS), coloured with orcein for LM visualization of the elastic network, or immunolabelled with antibodies to collagen type IV (rabbit polyclonal, diluted 1:200; Rockland Inc., Gilbertsville, PA, USA), to profilaggrin/filaggrin (AKH1 mouse monoclonal, diluted 1:20; Biomedical Technologies, Staughton, MA, USA), and to aquaporin-3 (rabbit polyclonal, diluted 1:100; AbCys, Paris, France) using biotin-streptavidin-peroxidase amplification method (LSAB 2 kit; Dako, Carpinteria, CA, USA). The sections were pretreated with 0.05% trypsin in PBS pH 7.2 (15 min, 37°C) prior to staining with the antibody to collagen IV.

For transmission EM, the standard embedding in Epon was preceded by fixation with 2% glutaraldehyde in 0.4 M sodium cacodylate buffer, postfixation in 1% osmium tetroxide and dehydration in graded ethanol series. Ultrathin sections were counterstained with lead citrate and uranyl acetate.

## Semi-quantitative evaluation of the elastic fibre network in the papillary dermis

Elastic fibres in the papillary dermis of each EM biopsy were observed in at least 10 consecutive observation fields at ×6000 magnification and photographed. Morphology of the elastic fibres was noted and the structures were qualified as 'composite' when the electron dense microfibrillar component was embedded in the lighter homogeneous material corresponding to elastin, typical of normal elastic fibre network. The 'fragmented' elastic fibres were characterized by the virtual absence of the electron lucent ground substance, leaving the fibrillar bundles disorganized. The presence of the 'composite' fibres was assessed semi-quantitatively in a double-blind manner by two observers and scored: ++++ (predominant fibre type), +++ (numerous), ++ (frequent), + (rare) or 0 (absent). A similar evaluation was performed on orcein-stained biopsies at the LM level. Here, the presence, density and continuity of fine oxytalan fibres spanning the space between the dermal-epidermal junction and superficial elaunin arches at the limit between the papillary and reticular dermis were scored (++++ = numerous and well organized, +++ = frequent, ++ = infrequent and poorly organised, + = rare, or 0 = absent) on at least five tissue sections per biopsy.

The scores from the biopsies taken before and after 6 months of treatment were paired for each site and the assignment to test or control side was revealed for further analysis. The volunteers were classified according to the degree of improvement taking into account both LM and EM scores and subdivided into three groups: no significant change, slight and marked improvement. In order to compare these morphological results with the functional findings, mean elasticity values measured with the cutometer were calculated for the subjects within each group and expressed as mean percent of improvement versus initial values.


 

## Results

### Clinical and biometric findings indicate a marked improvement after topical treatment with a combination of vitamin C and madecassoside

Significant differences were observed in the global score before and after treatment, starting as early as 3 months after the application of the vitamin C/madecassoside cream ($P < 0.0001$). After 6 months of treatment, the global score decreased in all the treated volunteers, with an improvement of at least 23.5% (mean = 29.5%; with a reduction in the global score from $32.7 \pm 2.6$ to $23.1 \pm 3.0$, mean ± SD). These differences observed on the face (Supplementary Fig. S1) were confirmed by the score assessed on the neckline, where the differences between the active and control creams were somewhat less pronounced but significant ($P < 0.05$ at 3 months). The global score improved progressively from $28.1 \pm 3.2$ at the baseline to $18.1 \pm 3.0$ after 6 months of treatment with the tested cream. On the control side, a slight improvement, although statistically not significant, was also observed (reduction in the global score from $27.8 \pm 2.6$ to $23.2 \pm 2.8$).

According to the dermatologist's assessment, the parameters demonstrating statistically significant improvement (with regard to the side treated with control cream) included hydration, roughness, laxity, suppleness, wrinkles, radiance and pigmented spots. Self-assessment by the volunteers also disclosed a statistically significant and beneficial evolution of most of these clinical items following topical application of the cream (Table S1).

Biometric assessments indicated a significant increase (versus the baseline value) of skin hydration (corneometer; from $55 \pm 9$ to $74 \pm 8$, $P < 0.0001$) and a significant reduction in the 'crow's feet' volume (fringe analysis; from $1.8 \pm 1.1$ mm$^3$ to $1.5 \pm 1.0$ mm$^3$, $P < 0.002$) as early as after 3 months of treatment with vitamin C/madecassoside. After 6 months of daily applications, a significant increase in cutaneous elasticity was observed (Ur/Ue cutometer values; from $0.68 \pm 0.11$ to $0.83 \pm 0.15$, $P < 0.0001$).

### Topical treatment with the vitamin C and madecassoside association induces re-structuring of the actinically damaged papillary dermis

Orcein staining, which permits visualization of the elastic fibre network, demonstrated the existence of severe to medium elastotic changes in all the samples before treatment. There was no significant modification in the skin morphology after treatment with the control cream. However, the test cream induced the appearance of new, fine elastic fibres of the oxytalan type and in the elaunin type arches, located at the limit between the papillary and reticular dermis (Figs 2 and 3). These fibres showed a 'composite' ultrastructure, characteristic for a normal elastic network

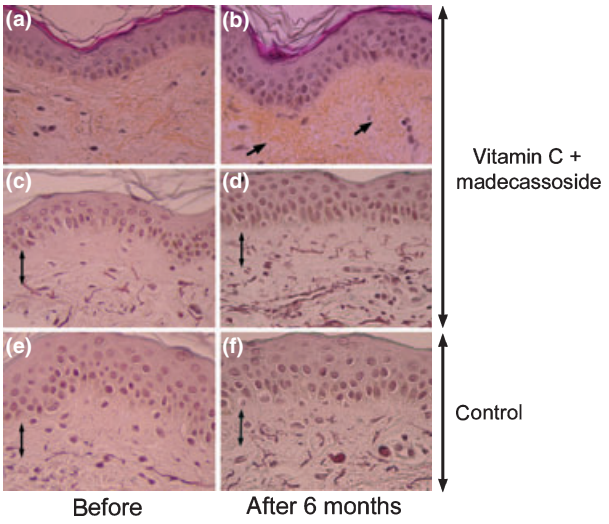


**Figure 2.** Actinic damage to the elastic network and its partial restoration after treatment with the vitamin C and madecassoside association as observed with light microscopy (LM). (a, b) Standard HPS staining. Dispersed collagen bundles in the papillary dermis become denser and better organized after 6 months of topical treatment with vitamin C/madecassoside (yellow staining, arrows); subject no. 8. (c–f) Orcein staining. Actinic damage in the papillary dermis and its replenishment after 6 months of treatment with the combination of vitamin C and madecassoside; subject no. 8. Two-way arrows indicate the thickness of the targeted dermis. (a–d) Vitamin C/madecassoside-treated site; (e, f) control site; (a, c, e) before treatment; (b, d, f) after treatment. ×400.

(Fig. 3). The changes were frequently observed close to fibroblasts displaying morphological signs of cell activation and fibre production. In many volunteers, initially dispersed and poorly structured papillary dermis became more compact and filled with multidirectional bundles of collagen fibres after treatment. A slight modulation of the extent of distribution of aquaporin-3 and filaggrin/profilaggrin, two molecules potentially involved in the stratum corneum hydration, could sometimes be observed on both sides, after treatment with both creams (Fig. S2). No change in collagen IV expression could be detected immunohistochemically (data not shown). In all the cases the basement membrane showed a continuous linear staining, before and after the treatment.

### The observed morphological changes corroborate the functional evaluation of skin elasticity

Fourteen volunteers were evaluated semi-quantitatively with LM and EM (one dropout for technical reasons). When subjects were classified into groups according to the degree of improvement in the elastic tissue structure, it turned out that 6 of 14 were good responders, three were fairly good responders, whereas five showed no significant change (Table 1). Mean percent of functional improvement in skin elasticity (Ur/Ue cutometry values measured

 

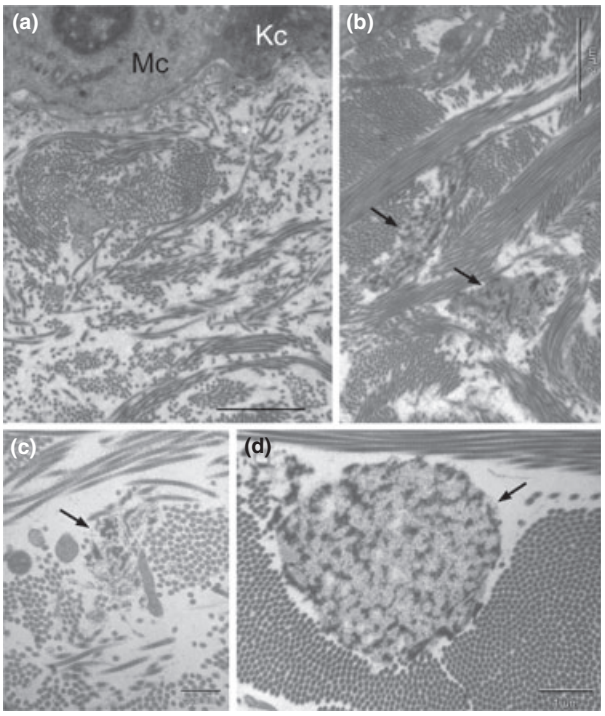


**Figure 3.** Ultrastructural changes in the papillary dermis. Elastic fibres, which were initially disorganized and infrequent, reappear in the actinically damaged papillary dermis and present the 'composite' morphology after 6 months of topical applications of vitamin C and madecassoside. The collagen network appears also better structured and compact. Transmission EM, subject no. 14. (a) Persisting severe actinic damage on the control side. Note the low density of collagen and absence of elastic fibres. (b) Dermal re-structuring with the association of vitamin C and madecassoside. The composite elastic fibres (arrows) are frequent in the papillary dermis, the latter being composed of multidirectional, interwoven, compact collagen bundles. A higher power micrograph illustrates elastic fibre fragmentation (c) and re-composition (d), respectively (arrows). Mc, melanocyte; Kc, keratinocyte. Bars = 2 $\mu$m in (a) and (b), 0.5 $\mu$m in (c) and 1 $\mu$m in (d).

**Table 1.** Effects of 6 months of topical treatment with a combination of vitamin C and madecassoside

| Subject no. (age) | Papillary dermis of a forearm before/after treatment: LM: structured, orcein-stained network | Papillary dermis of a forearm before/after treatment: EM: 'composite' elastic fibres | Skin elasticity (face cutometry; Ur/Ue) Mean % improvement vs. initial value | Classification according to the obtained results |
|---|---|---|---|---|
| 8 (48) | +/++++ | +/++++ | 56.8 | Good responders |
| 3 (46) | +/+++ | ++/++++ | | |
| 6 (55) | ++/+++ | +/+++ | | |
| 5 (54) | +/++ | 0/++ | | |
| 4 (53) | +/++ | 0/++ | | |
| 15 (51) | +/+++ | +/+++ | | |
| 20 (48) | ++/++ | ++/+++ | 21.7 | Weak responders |
| 14 (57) | +/++ | +/+++ | | |
| 17 (46) | +/++ | +/++ | | |
| 19 (56) | +/++ | 0/+ | 4.4 | No significant change |
| 10 (53) | +/+ | 0/+ | | |
| 16 (45) | +/+ | 0/+ | | |
| 13 (57) | +/+ | 0/+ | | |
| 11 (45) | +/+ | +/+ | | |

LM (light microscopy): + to ++++; degree of visualization of the structured elastic network in the papillary dermis.
EM (electron microscopy): 0 to ++++; quantity of the 'composite' elastic fibres visualized in the papillary dermis.
Cutometry (Ur/Ue): the degree of improvement for each group, of good, medium and non-responders, is expressed as the mean of individual increases in skin elasticity (for each subject, percent increase compared with the initial value).

on the face) in the volunteers belonging to the three groups fully corroborated the morphological data and read 56.8%, 21.7%, and 4.4% respectively. Thus, re-appearance of the normally structured superficial elastic network may explain the functional improvement of skin elasticity in subjects treated with the association of vitamin C and madecassoside.

## Discussion

We have previously demonstrated that prolonged topical treatment with vitamin C partially restores some of the clinical and structural signs of cutaneous ageing (12,13). In the present study, we were looking for similar and supplementary effects using a formulation containing vitamin C and madecassoside.

The so-called anti-ageing products should ideally combine components aiming at prevention of the environment-induced changes with agents stimulating cell metabolism, synthesis and re-structuring of the dermal fibrous matrix. However, the outcome of tissue and cell 'rejuvenation' is also partially dependent on action against already installed age-related changes. Partial removal of the damaged elements could constitute an important complementary mechanism of improvement of the physical characteristics of aged skin. Such a combined action may be reached through the local increase in antioxidants and enzyme-regulating agents. Indeed, vitamin C induces collagen production in fibroblasts, partially through the modulation of collagen-synthesizing enzymes (19,20). It also controls collagen-degrading enzymes like matrix metalloproteinase-2 (21), whereas tissue concentration of ascorbic acid is significantly reduced in aged and photoaged skin (22–24). Furthermore, the beneficial effect of vitamin C on the dermal-epidermal junction morphogenesis


 

has been demonstrated in an *in vitro* reconstructed human skin model (25). These experimental data indicate the potential role of vitamin C in the improvement or regeneration of this zone, which is essential for skin cohesion, resistance to mechanical stress and exchanges between the epidermis and the dermis. The expression of collagen IV in the epidermal basement membrane remained unchanged throughout our study, which is not surprising because no morphologically detectable damage to this zone could be observed in the initial biopsies of the aged skin.

All the reported data are in favour of the use of topical vitamin C supplementation and suggests that the 5% ascorbic acid present in the studied formulation was at least partially responsible for the observed structural remodelling of the dermal collagen and elastic tissue networks. Accumulating laboratory evidence indicates that many botanical agents with antioxidant properties exert anti-photoageing effects in the skin (26). Some of the plant extracts seem to improve skin hydration through a mechanism involving aquaporin-3 upregulation in keratinocyte and filaggrin processing (27). Although no detectable changes in aquaporin-3 expression and profilaggrin/filaggrin distribution could be detected immunohistochemically in our study, the principle of association of an antioxidant like vitamin C with an active ingredient of botanical origin like madecassoside, working through different mechanisms, seems to be an attractive one. Madecassoside has been shown to act on collagen synthesis *in vitro* (14,16,17) and *in vivo* (15), apparently by activating the Smad signalling pathway (28). The association of both active components proved to be highly beneficial from clinical, morphological and functional points of view, as demonstrated in our study. It remains, however, to be elucidated as to whether the two compounds exert a synergistic or just an additive effect causing the remodelling of the superficial dermis.

Our clinical data and those described *in vivo* and *in vitro* in the literature were supported by the biometrological assessment, especially the results of cutometry. To study skin mechanical properties, many devices can be used, which create different types of constraints, e.g. torsion, suction, compression and stretching. Among them, twistometry and cutometry have already shown their relevance in studies of skin ageing (29). With both techniques, the main pertinent parameters for assessing skin elasticity are skin resistance to deformation and skin ability to recover its initial state after deformation. In practice, in clinical studies, skin elasticity is expressed as the ratio between immediate recovery after release and initial deformation (Ur/Ue). This parameter decreases gradually with ageing, mainly because of the alteration in elastic fibres (30). In this study, the cutometer demonstrated an improvement in skin elasticity after treatment, which was well correlated with the improvement in elastic tissue structure. Moreover, a significant decrease in the 'crow's feet' wrinkles was observed after 3 months of treatment. This measurement was performed with a device developed in Besançon to study the skin relief directly *in vivo*. Thanks to its great sensitivity (resolution of 5 $\mu$m) and its fast data input (about 500 ms per picture) this technique does not require a fastidious preparation of silicone replicas of the skin surface (18).

Our ultrastructural and LM observations fully corroborate and partially explain the positive results of the clinical and biomechanical/functional investigations in subjects treated with a combination of vitamin C and madecassoside. The control hydrating cream with no active ingredients was well accepted by the studied subjects and it provided some degree of improvement in the patients' subjective scores and in the clinical and biometric evaluations made by the investigators (Table S1). However, the benefit from the treatment with vitamin C/madecassoside was significantly higher in all cases except the results of corneometry. As this latter method measures essentially the degree of SC hydration, we suppose that the control hydrating cream was sufficient in improving this parameter on the control side, rendering the difference against the active compound negligible. The general state of the skin at any point of the ageing process depends on the balance between the induced alterations and the repair processes. Indeed human skin preserves a significant repair capacity up to the age of 50 allowing a moderate self-restoration in the case of relief from causative agents (3,31). In this context, the combined action of vitamin C and madecassoside may have contributed to a shift from the balance towards skin repair.

When dealing with chronic actinic skin damage, efforts should be made not only in the domain of protection from sun exposure but also towards the stimulation of the local skin re-structuring mechanisms. The latter approach offers both preventive and therapeutic virtues.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Examples of favourable clinical results obtained after 3 and 6 months of treatment with vitamin C/madecassoside association. (a–c) Volunteer no. 1 (no biopsy); (d–f) volunteer no. 8. The most remarkable improvement consists of a significant attenuation of fine and deep wrinkles. a, d = before treatment; b, e = after 3 months of vitamin C/madecassoside application; c, f = at the end of the trial.

**Figure S2.** Immunohistochemical approach does not show any consistent modification of the expression of profilaggrin/filaggrin or aquaporin-3, two molecules involved in epidermal hydration. Six-month treatment with vitamin C/madecassoside cream (b, d) did not affect the distribution pattern of filaggrin/pro-filaggrin (a, b) and aquaporin-

 

3 (c, d), when compared with the distribution before the treatment (a, c) in the same volunteer. ×400.

**Table S1.** Minimal and mean improvement observed clinically after 6 months of treatment compared with the results of corneometry.

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