CutiGuard CLR™

A refined approach to the
first signs of skin aging

for fine cosmetics · since 1926

CutiGuard CLR™

A refined approach to the first signs of skin aging

CONTENTS

CutiGuard CLR™

Dosage: 3.0%
pH range: 3.0–9.0
INCI Name: Betaine, Sucrose, Hydrolyzed Rhodophyceae Extract, Water

CutiGuard CLR™ is unpreserved.

For more information, such as formulations or natural certifications, scan or click QR code:

SUMMARY

Based on the extremophile red alga *Galdieria sulphuraria*, CutiGuard CLR™ constitutes a refined approach to the first signs of skin aging. It transfers the adaptivity and resilience of *Galdieria sulphuraria* to a highly effective, natural active ingredient that protects the skin in stressful environments.

Skin aging starts in the skin cells, which, during a lifetime, accumulate damage. Extrinsic and intrinsic factors add damage, leading to prolonged inflammation and cell senescence. CutiGuard CLR™ reduces the number of senescent cells and associated inflammatory and destructive mediators which lead to visible skin aging. It thus protects skin against premature aging. CutiGuard CLR™ also reduces wrinkles and significantly improves skin smoothness as well as the evenness of skin color – three crucial factors for a healthy and young appearance.

With its proactive and preventive approach, CutiGuard CLR™ is extremely suitable for modern aging consumers. It slows down the skin aging process and helps to avoid the development of the first signs of skin aging.

APPLICATION

- Anti-wrinkle, firming, smoothing
- Skin regeneration
- Skin protection

■ tested ❑ recommended

CutiGuard CLR™

THE AGE OF CHANGE

Anti-aging skincare is not what it used to be. For the cosmetic industry, it is still the most important type of product financially within the skincare segment, but in recent years the concept of anti-aging skincare has changed rapidly in most parts of the world. The "anti" in anti-aging skincare is essentially outdated. Skincare consumers do not have an "anti-way of thinking" about their skin and the products they use to take care of their skin. They are much more positive in their attitudes.

Most skincare consumers have stopped believing that skincare products potentially offer a "miracle in a jar." They do not believe that a cosmetic product is able to miraculously lead to the disappearance of wrinkles or any other aging-related skin features. Especially not when the aging process has been going on for a long time. "Anti-aging" skincare is still booming, though, but it needs a dramatically different approach. Literally, "anti" can be replaced by "pro", or "healthy" or "successful" or "better". Better aging is what the skincare consumer is most interested in. "Looking the best version of yourself" while accepting that the aging process is unavoidable is the new modern credo.

Not "anti" but better! What, though?

"Better aging" skincare consumers are proactive and preventive in their attitudes. "It is better to prevent than to cure." People start using anti-aging skincare products at a far younger age than previously. They want to prevent, or at least slow down, the skin aging process. In this effort they especially focus on the visible signs of aging. Skincare products which provide skin protection and the ability to "help the skin to help itself" in the aging process are of particular interest to them. Herein lies an enormous challenge for the cosmetic industry.

The first part of providing a fitting answer to the challenge is the recognition of the exact features which make skin look older. Anti-aging skincare products have always focused on wrinkles, skin firmness and uneven pigmentation. The first two topics are indeed important for "how old skin looks," but the factor pigmentation is rather more nuanced, depending on the type of skin you have. Pigmentation plays a role in how old the skin looks, but zooming out is necessary. Apart from melanin, there are many more chromophores in the skin which play a role in the (unevenness of) skin coloration. With aging these chromophores can play a role in how old skin looks.

Three skin aging drivers

A next step in providing the consumer with satisfactory products for their aging skin is understanding the factors which cause the visible signs of skin aging and, in combination with that, the exact biological mechanisms through which they bring about these problems. There are three factors which play a role in skin aging. First of all, time. Skin aging, for the biggest part, starts in the skin cells. In simplified terms: over time, skin cells lose their youthful functionalities and abilities. They become less proliferative and lose their capacity to react to challenges coming from the outside and inside of the body.

These outside (extrinsic) and inside (intrinsic) influences constitute factors number two and three. Sunlight is a well-known extrinsic factor which influences the skin aging process. It is said that 80% of all visible signs of skin aging are caused by UV light ("photoaging"). Modern science has shown that infrared and visible light also accelerate the skin aging process. Other factors, such as pollution and smoking, are further examples of extrinsic factors that influence the skin aging process.

Intrinsic factors have long been, and are still, underrated as a source of skin aging. Psychological stress, lack of sleep, diet, and many more such factors can have a strong impact, for instance on the hormonal composition within our body. "Stress hormones," produced inside the human body, for example, are infamous for having a large negative impact on skin. For most people it is a well-known fact that, when a person is stressed, her or his skin suffers from it in most cases.

Understanding the mechanisms

The three factors of skin aging, time, intrinsic and extrinsic factors lead to the development of visible signs of skin aging. The next questions are, how do these visible signs of skin develop mechanistically, and what influences do these factors have on the biological mechanical processes leading to the formation of wrinkles, unevenness in skin color and loss of skin firmness and elasticity? As mentioned above, for the largest part, skin aging starts in the skin cells. As a result of the three factors of skin aging, the skin cells "change" and, downstream of this, the architecture of skin is changed. This leads to the formation of wrinkles and loss of firmness and elasticity. As described above, unevenness of skin color is only partly due to pigmentation (although this depends on ethnicity). What is clear, though, is that there is an important aging-induced inflammatory aspect to this visible skin aging-feature.

In finding a solution to the problem of the occurrence of visible signs of skin aging and, thus, also an effective active ingredient, the cellular aspects of skin aging need to be fully understood. The main skin cells in relation to skin aging are the keratinocytes of the epidermis and the fibroblasts of the dermis. In time, under the influence of extrinsic and intrinsic factors, these cells become damaged and stressed. Typical cellular features of skin aging are an accumulation of nuclear DNA damage, a prolonged and unsuccessful DNA damage response, and oxidative stress. With age, an increasing number of these skin cells are not able to recover from these problems ("cellular aging").

Cell biology of senescence

Aged cells will then enter a process in which they turn senescent. The senescent skin cells are not able to proliferate, but their well-developed anti-apoptotic mechanisms ensure that they stay alive. Senescent cells remain metabolically strongly active. They constantly produce a number of inflammatory mediators and proteases, such as Interleukin-6 (IL-6), IL-8 and matrix metalloproteinases (MMPs), such as MMP-1. The family of detrimental mediators and proteases secreted by senescent cells is summarily called the senescence associated secretory phenotype, or SASP. The members of the SASP are in essence an important cause of the deterioration of skin during aging and play a major role in the development of the visible signs of skin aging.

For a cosmetic active ingredient fitting the needs of the modern "anti-aging" skincare consumer, i.e., slowing down the aging process, it is opportune to act on the SASP and the senescent cells themselves. It is also important, however, to influence the process with which skin cells turn senescent. The main cell biological player in this process is GATA4 (GATA binding protein 4). GATA4 is the main driver of cell senescence. It is a transcription factor which is activated in irreversibly damaged cells. In this role, GATA4 leads to the expression of (inflammatory) genes which, after translation into a protein or enzyme, push the process of senescence forward.

GATA4 is an important focus for a cosmetic active ingredient. After complexation with p62, GATA4 can be degraded through autophagy. An active ingredient which can elevate p62 and autophagy, can, therefore, reduce GATA4 and, thus, also reduce senescence (which can be determined by an analysis of the senescence marker SA-ß-galactosidase) and the production of the members of the SASP. There is another important cell biological feature to take into consideration, though: HMGB1 (High Mobility Group Box-1).

HMGB1 is a nuclear protein which, in healthy and functional cells, resides inside the cell nucleus and plays an important role in healthy functionality. When skin cells become damaged, HMGB1 is secreted from the cells and enters the extracellular space. From this point onward, HMGB1 plays a detrimental role. Through the interaction with receptors on neighboring skin cells it initiates the production of a large group of inflammatory cytokines. Here too, IL-6 and IL-8 become main players, induced by HMGB1. HMGB1 is not just excreted by damaged cells, it is also a typical marker for senescent cells. It is important, therefore, to be put in the context of accelerated skin aging and the development of the visible signs of skin aging.

An active ingredient which acts on GATA4 (through p62 and autophagy), senescence, SASP and HMGB1 will slow down the aging process, the main interest of the anti-aging skincare consumer. Another important need of this consumer should also be taken into consideration. Being preventive and proactive in her attitude, she finds it important to slow down the aging process and prolong skin youthfulness, but, on the other hand, realizes that this process cannot be stopped. She realizes that at some point wrinkles will start to occur. These wrinkles will first be visible as persistent fine, rather superficial, lines. Although she realizes that it is not possible to make deep wrinkles disappear with skincare products, the anti-aging skincare consumer does feel the need to be able to reduce fine lines and superficial wrinkles once they start to become visible.

The active ingredient, therefore, needs to address those cellular features which allow for the production of important molecules in reducing the fine lines and superficial wrinkles. Cell energy (ATP) is an interesting indicator for cell functionality, but the ability for fibroblasts (in interaction with keratinocytes) to produce, for instance, Collagen Type I and Hyaluronic acid, with and without the negative impact of the three factors of skin aging mentioned above, is another important focus.

INTRODUCING: CutiGuard CLR™

On the basis of the above-described approaches and philosophy, CLR developed CutiGuard CLR™ (INCI: Betaine, Sucrose, Hydrolyzed Rhodophyceae Extract, Water). CutiGuard CLR™ is based on *Galdieria sulphuraria*, a red alga which can best be described as an extreme extremophile. It can be found in hot volcanic sulfur springs but can just as well thrive among toxic metals like arsenic and mercury. *Galdieria sulphuraria* is an extremely adaptive and resilient species. In its quest to maintain viable even under the most hostile of environments, it can go through processes of "horizontal gene transfer," where resilience genes from other unicellular species, archaea, are transferred to *Galdieria sulphuraria* to make it even more extreme in its extremophilic existence.

A cleverly designed, extremely efficient and sustainable fermentation process leads to a highly efficacious extract of *Galdieria sulphuraria*, which is at the core of CutiGuard CLR™. The ionic mixture of betaine and sucrose allows the cells of *Galdieria sulphuraria* to be broken down for CutiGuard CLR™ to provide all the necessary ingredients from *Galdieria sulphuraria*, including those from inside the cells which the skin needs. The skin and skin cells are supported and, with the help of CutiGuard CLR™, become more resilient and adaptive themselves.

EFFICACY STUDIES – *in vitro* assays

With CutiGuard CLR™, *in vitro* studies were performed with a focus on GATA4 (and p62 and autophagy), senescence, SASP, HMGB1, as well as cell energy (ATP), Collagen Type I and Hyaluronic acid. These studies were performed under the influence of time, extrinsic (UV) and intrinsic stress. For mimicking the role of intrinsic stress on these aging-relevant skin features, the focus was on cortisol. Cortisol, when produced in higher concentrations, is a well-known stress hormone. It is also well known to induce nuclear DNA damage, increase senescence, SASP and oxidative stress, underlining the importance of intrinsic factors in skin aging.

The cell biological proof-of-concept of an active ingredient is important when relevant to the challenges at hand, but clinical (*in vivo*) proof is of the essence. Visible skin aging encompasses lines and wrinkles, unevenness of skin coloration and loss of firmness and elasticity, where skin sagging ("ptosis") becomes visible. The influence of the active ingredient on these three skin issues needs to be proven in a relevant context with a relevant study design on volunteers who are part of the target audience of the ingredient.

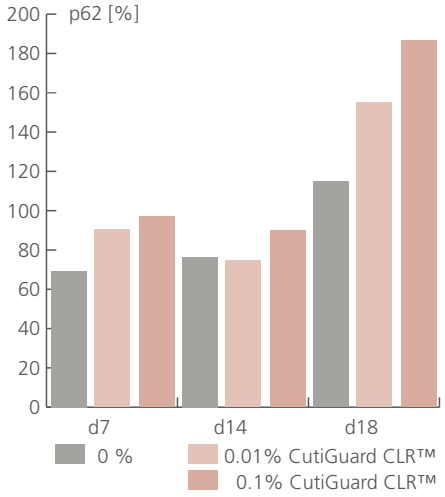

Fig. 1: Influence on expression of p62

P62

A study was performed on HaCaT keratinocytes, which were aged for a total of 18 days under chronic stress of combined hydrocortisone (1 µM) and TNFα (1 ng/ml). Method of measurement: CycLex Total p62 ELISA (Biozol), MBL-CY-7055, 450 /570 nm. Expression of p62 at day 0, untreated and non-stressed, was set at 100%.

P62 (also known as SQSTM1) is a classic selective autophagy receptor. As an autophagy substrate, it delivers proteins bound to it to the autophagosome for autophagocytotic degradation. In this role and relevant for CutiGuard CLR™, it binds to GATA4 and initiates autophagocytotic degradation of GATA4.

During aging, expression of p62 has been described to be reduced. In analogy, an inverse relationship is also described to exist between p62 expression and quantity of senescent cells. As GATA4 expression leads to an increased quantity of senescent cells and autophagocytotic degradation of GATA4 is p62-dependent, this inverse relationship is comprehensible.

Induction of p62 expression is an important approach toward the reduction of GATA4-induced cellular senescence and skin aging.

Results

At days 7, 14 and 18 it is shown that CutiGuard CLR™, at different concentrations, induces the expression of p62 (Fig. 1).

Autophagy

A study was performed on HaCaT keratinocytes which were incubated for 48 hours with 2 µM Chloroquine and with and without CutiGuard CLR™ at different concentrations. Method of measurement: LC3B (D11) XP® Rabbit mAb (NEB; 3868) + Anti-Rabbit IgG (H+L), F(ab')2 Fragment (Alexa®488) (NEB; 4412), 485/535 nm. Marker for autophagy is LC3B, which is embedded in the autophagosomes membranes. Chloroquine disallows for lysosomal degradation of autophagosomes and, therefore, plays an important role in the quantification of autophagosomes through LC3B and activity of the autophagy machinery. Non-treated control was set at 100%.

Autophagy is important in the degradation of GATA4, bound to p62 as a specific autophagy receptor. GATA4 induces cellular senescence. Autophagy plays an important role in the reduction of the quantity of senescent cells.

Results

At different concentration, CutiGuard CLR™ strongly induces autophagy in HaCaT keratinocytes (Fig. 2). These results, together with the results obtained on p62, indicate that CutiGuard CLR™ should induce the autophagocytotic degradation of GATA4 and, therefore, the number of skin cells going into senescence.

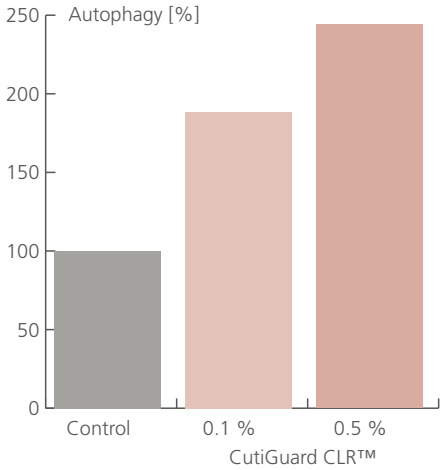

Fig. 2: Influence on autophagy as measured through LC3B

GATA4

A study was performed on HaCaT keratinocytes, which were aged for a total of 18 days with and without chronic stress of combined hydrocortisone (1 µM) and TNFα (1 ng/ml). Method of measurement: Mouse/Human Phospho-GATA4 (Ser262) (DNA-Binding Phosphorylation ELISA) (Biozol), LS-F877, 450/570 nm. GATA4 expression of control cells, not treated with CutiGuard CLR™, at day 18 was set at 100%.

GATA4 (GATA Binding Protein 4) induces cellular senescence and can be broken down through the cellular autophagy machinery where p62 acts as a specific autophagy receptor for GATA4. Autophagy and p62 expression could both be activated with CutiGuard CLR™, explaining the results obtained on GATA4 degradation.

Results

Results were obtained at day 18 of the experiment. The HaCaT keratinocytes which were not stressed but treated with CutiGuard CLR™ show reduced presence of GATA4 (Fig.3). This is also the case for the cells which were stressed with hydrocortisone and TNFα, but in this case, the reduction of GATA4 is clearly more pronounced. CutiGuard CLR™ indeed induces degradation of GATA4.

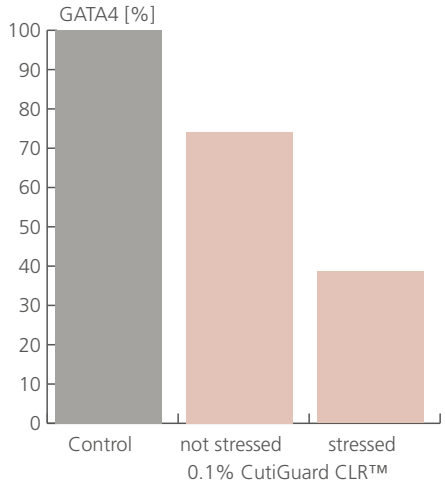

Fig. 3: Influence on GATA4, through autophagocytotic degradation

Senescence

A study was performed on normal human primary fibroblasts, which were aged for a total of 19 weeks under chronic stress of combined hydrocortisone (1 µM) and TNFα (1 ng/ml). Method of measurement: Senescence-β-Galactosidase Staining Kit (Cell Signaling Technology), 9860S, 570 nm. Non-treated control, at the different time-points, was set at 100%.

Since senescent cells express a large quantity of pro-inflammatory mediators and destructive proteases, together described as the senescence-associated secretory phenotype (SASP) as well as strongly pro-inflammatory HMGB1 (High Mobility Group Box-1), they play a key role as a motor behind skin aging. p62-enabled autophagocytotic degradation of GATA4 is an important approach in reducing the number of senescent cells. To elegantly quantify the activity of CutiGuard CLR™, fibroblasts were utilized, as these are long-lived and their aging process can be measured reliably. As a marker of cellular senescence, SA (senescence-associated)-ß-Galactosidase was chosen.

Results

With increased age of the fibroblasts, it could be clearly shown that CutiGuard CLR™ increasingly reduces the number of senescent cells (Fig. 4). These results are in line with the results obtained on p62, GATA4 and autophagy, and provide robust proof of the important activity of CutiGuard CLR™ in skin aging.

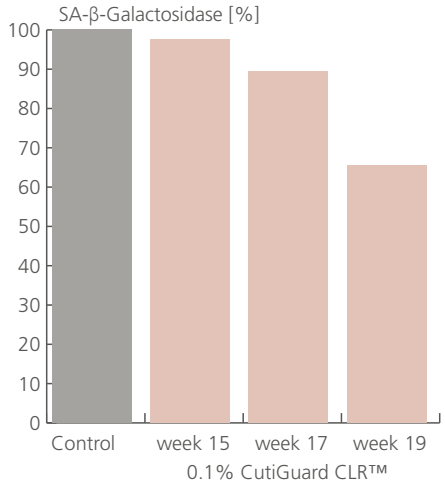

Fig. 4: Influence on senescence as measured through SA-β-Galactosidase

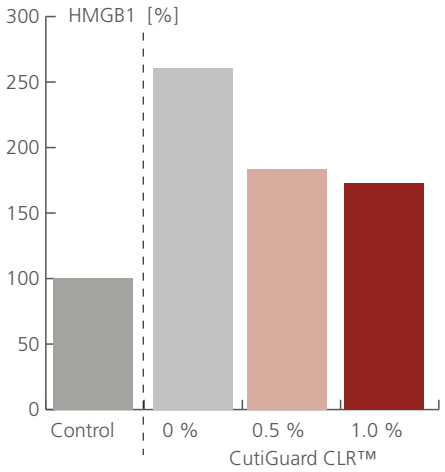

Fig. 5: Influence on HMGB1 after stress with TNFα

HMGB1

HMGB1 (high mobility group box-1) is expressed by both damaged and senescent cells in large quantities. It is, in fact, recognized as the second senescence-associated initiator of aging-related deterioration of skin, second to the SASP. HMGB1 is pro-inflammatory, in the sense that it can activate transcription factors such as NF-κB, which induces the production of a large family of inflammatory mediators, of which cytokines like IL-6 and -8 are a member. Reduction of HMGB1 expression is therefore an important approach in slowing down the skin aging process.

A study was performed on HaCaT keratinocytes which were incubated for 24 hours with and without CutiGuard CLR™ and with or without TNFα (1 ng/ml) as an initiator of HMGB1 expression. Method of measurement: High Mobility Group Box1 Protein (IBL International), ST51011, 450/600 nm. Non-treated and non-stressed control was set at 100%.

Results

The treatment with TNFα strongly induces the expression of HMGB1 (Fig. 5). The treatment with different concentrations of CutiGuard CLR™, however, led to a clear decrease of HMGB1 expression. These results show that CutiGuard CLR™ has a potent and relevant effect on this skin aging–inducing pathway.

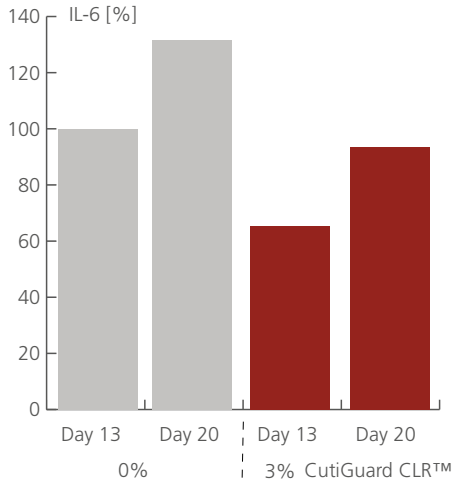

Fig. 6a: Influence on IL-6 expression, aged full skin models

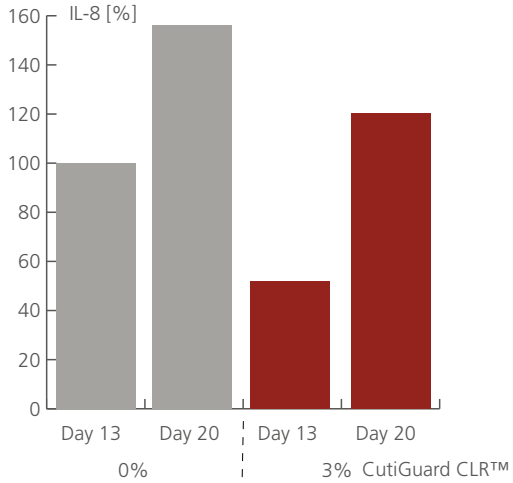

Fig. 6b: Influence on IL-8 expression, aged full skin models

Senescence-associated secretory phenotype (SASP)

Interleukin (IL)-6 and -8 as well as Pro-MMP-1 are important representatives of the SASP. Several different experiments were performed to assess the effect of CutiGuard CLR™ on the expression of these mediators. As the number of senescent cells could be shown to be reduced with CutiGuard CLR™, it was expected that it could also reduce the expression of these SASP family members.

IL-6 and -8, experiment 1

A study was performed on aged full skin models (containing an epidermis with keratinocytes and a dermis with fibroblasts), which were aged over a period of 20 days. The skin models were treated with and without CutiGuard CLR™. Method of measurement: Human Interleukin-6 (Biotechne (R&D)), DY206, 450/570 nm and Human Interleukin-8 (Bio-techne (R&D)), DY208, 450/570 nm, respectively. Values obtained for the non-treated control on day 13 of incubation were set at 100%.

Results

In both cases it could be shown that both at day 13 and day 20, the expression of these inflammatory cytokines could be clearly reduced (Fig.6a (IL-6) and Fig.6b (IL-8)). What can also be concluded from the experiments is that, with time and as was expected, the expression of IL-6 and -8 became increased (at day 20). CutiGuard CLR™, however, was able to strongly control their expression and keep them around or, in the case of IL-6, below the baseline level (level of untreated control at day 13).

IL-6 and -8, experiment 2

Other experiments were performed with full skin models. In these cases the skin models were stressed and artificially aged using UV irradiation (1 J UVA/UVB). As a well-known accelerator of skin aging ("photoaging"), UV radiation is known to strongly induce senescence and, therefore, the production of SASP members, such as IL-6 and -8. Method of measurement: Human Interleukin-6 (Bio-techne (R&D)), DY206, 450/570 nm and Human Interleukin-8 (Bio-techne (R&D)), DY208, 450/570 nm, respectively. Values obtained with untreated and non-stressed control at day 2 of the experiment were set at 100%.

Results

It could be clearly shown that in the course of the experiment, at day 7, the UV-irradiated, non-treated full skin models showed a higher expression of IL-6 and IL-8. In both cases, CutiGuard CLR™ was shown to reduce the expression of these mediators (Fig. 7a (IL-6) and Fig. 7b (IL-8)).

Both experiments 1 and 2 show that CutiGuard CLR™ reduces the expression of senescence-associated IL-6 and IL-8. These results strongly indicate that CutiGuard CLR™ helps in slowing down the skin aging process.

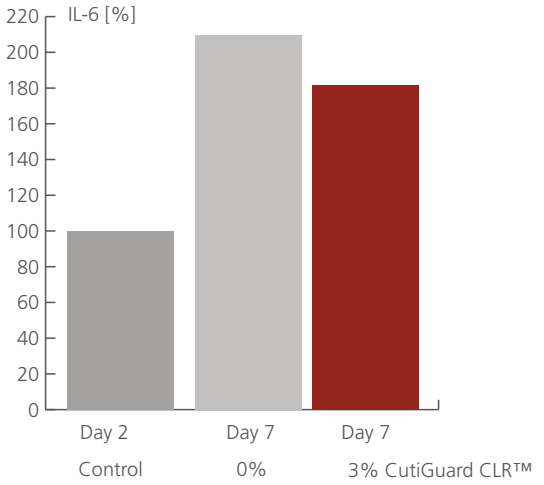

Fig. 7a: Influence on IL-6 expression, full skin models, irradiated with UV light

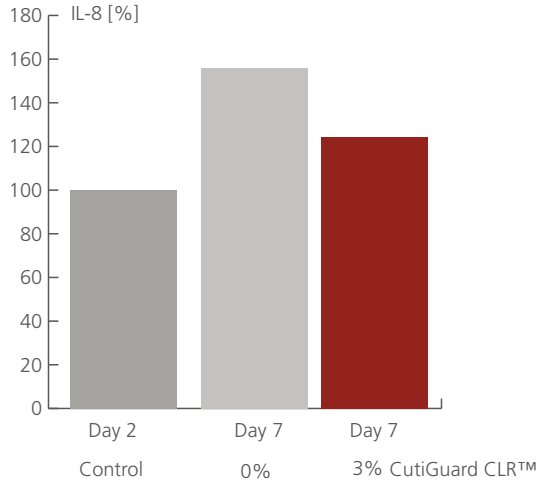

Fig. 7b: Influence on IL-8 expression, full skin models, irradiated with UV light

Pro-MMP-1

A study was performed on full skin models which were aged with and without chronic stress of UV-irradiation (1J UVA/UVB). Method of measurement: Human Pro-MMP-1 DuoSet ELISA (R&D Systems), DY900. Non-treated, non-stressed controls at day 2 were set at 100%.

Results

With and without UV stress, in the controls, Pro-MMP-1 expression was increased. Surprisingly, though, Pro-MMP-1 expression in the irradiated control was lower than that of the non-irradiated control. This might have to do with the timing of the sampling after the last irradiation with UV. What is clearly visible in the results, however, is that in both cases CutiGuard CLR™ was able to clearly reduce the expression of Pro-MMP-1 (Fig. 8).

In analogy with the experiments on IL-6 and -8 as well as the other relevant *in vitro* studies which were performed, CutiGuard CLR™ was shown to be able to act on the most important cellular pathways leading to skin aging.

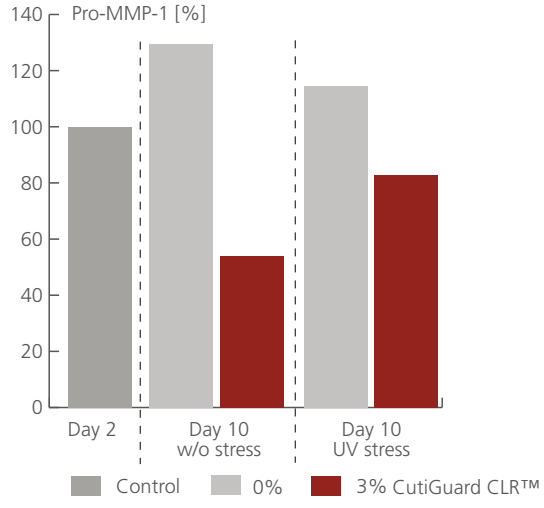

Fig. 8: Influence on Pro-MMP-1 expression, full skin models, irradiated with UV light

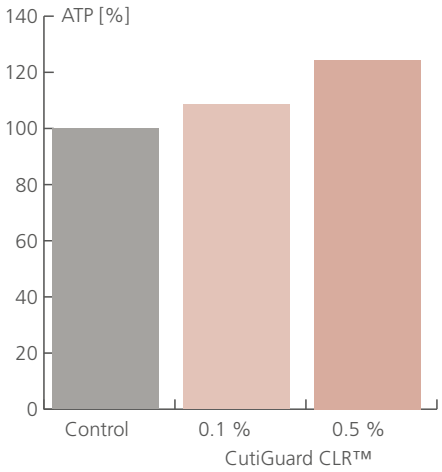

Fig. 9: Influence on cellular energy level

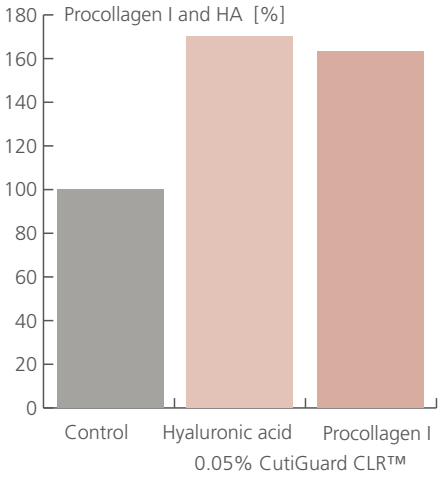

Fig. 10: Influence on expression of Hyaluronic acid and Procollagen I, under acute stress

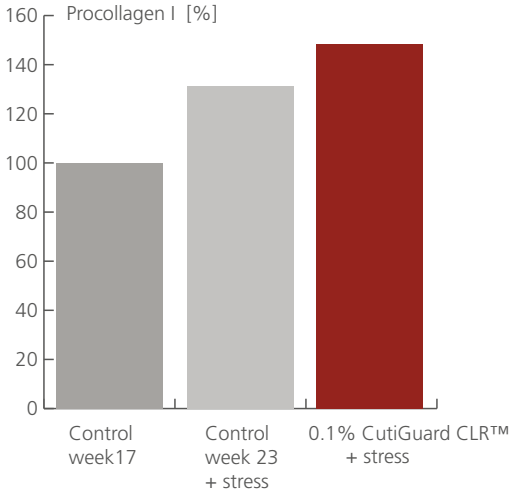

Fig. 11: Influence on expression of Procollagen I, under chronic stress

Cell energy

The results in the above-described experiments strongly indicate that CutiGuard CLR™ is able to slow down the skin aging process significantly. In order to assess whether CutiGuard CLR™ could also support skin cells in their regenerative action, where they constantly work on maintaining skin homeostasis and youthfulness, additional experiments were performed.

Cell energy (cellular ATP production) is an indicator of cellular activity. Cellular activity is reduced with aging. A study was performed on HaCaT keratinocytes to elucidate if CutiGuard CLR™ is able to increase cellular ATP production, i.e., energize these skin cells. The HaCaT cells were incubated with and without CutiGuard CLR™ for 48 hours. Method of measurement: ATPLite-M (Perkin Elmer), 6016941, luminescence. Non-treated control was set at 100%.

Results

At different concentrations, CutiGuard CLR™ was able to increase cellular energy (Fig. 9). This indicates that CutiGuard CLR™ can indeed activate skin cells and re-establish their youthful functionality. This was further investigated in subsequent studies on fibroblasts and full skin models.

Procollagen I and Hyaluronic acid

Both Procollagen I and Hyaluronic acid (HA) play pivotal roles in the mechanical properties and the topography of skin. With skin aging, their presence as well as their production are strongly reduced in the skin. This leads to a reduction of skin firmness and elasticity as well as skin sagging, but will also lead to the formation of wrinkles.

As described in the introduction of this brochure, consumers are interested in slowing down skin aging, but also in reducing the signs of skin aging as soon as they become visible. They are, therefore, interested in skincare products which include an activity on both features. Induction of Procollagen I and HA is of great interest, especially under stress conditions. Skin is under constant stress, which should be taken into consideration in obtaining consumer-relevant results.

Several experiments were performed:

Experiment 1: Procollagen I and HA

A study was performed on normal human dermal fibroblasts which were under acute stress of hydrocortisone (1 µM) and TNFα (1 ng/ml). These cells were incubated with and without CutiGuard CLR™ for 72 hours. Method of measurement: Pro-Collagen I alpha 1, DY6220-05; Hyaluronan ELISA, DY3614-05, (Bio-techne (R&D)), 450/570 nm. Non-treated and non-stressed control at t = 0 was set at 100%.

Results:

It could be shown that both the production of Procollagen I and HA were increased, despite the acute stress to which the fibroblasts were exposed (Fig. 10).

Experiment 2: Procollagen I

Another study was performed on normal human dermal fibroblasts. In this case, however, the fibroblasts were aged over a total of 23 weeks. The aging process took place with chronic stress of hydrocortisone (1 µM) and TNFα (1 ng/ml) and with and without CutiGuard CLR™. Method of measurement: Pro-Collagen I alpha 1, DY6220-05, (Bio-techne (R&D)), 450/570 nm. Non-treated control at week 17 was set at 100%.

Results

Somewhat surprisingly, with time the Procollagen I-production in the control cells which were not treated with CutiGuard CLR™ was elevated (Fig. 11). This result might originate from a cellular reaction by the fibroblasts to the stress to which they were exposed. In experiments which are not shown in this brochure it could be clearly confirmed that CutiGuard CLR™ was not a stress to any of the skin cells and models which were used in our studies. The incubation led to an important increase of Procollagen I expression, in analogy with the results obtained in experiment 1.

Experiment 3: Procollagen I

Yet another study was performed, this time on full skin models which were aged for 10 days, with and without UV stress (1 J UVA/UVB) and treated with and without CutiGuard CLR™. Method of measurement: Pro-Collagen I alpha 1, DY6220-05, (Bio-techne (R&D)), 450/570 nm. Results obtained on skin models which were non-treated and non-stressed at day 2 were set at 100%.

Results

The results obtained were in line with the expectations. The non-treated aged skin models which were not irradiated with UV light showed indeed a clearly reduced expression of Procollagen I, whereas those which were irradiated showed an even lower expression (Fig. 12). In both cases it could be shown, however, that CutiGuard CLR™ improves Procollagen I-production.

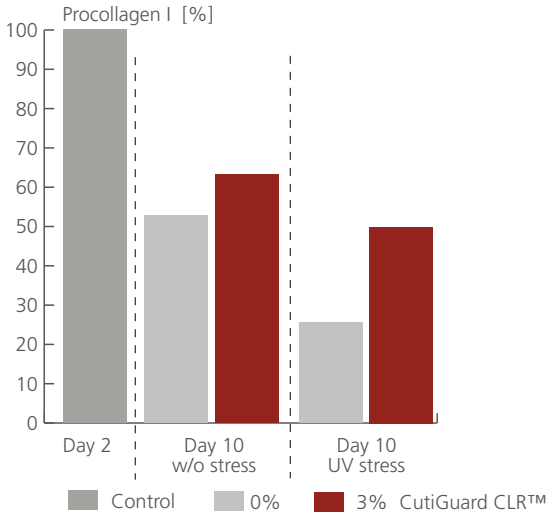

Fig. 12: Influence on expression of Procollagen I, with and w/o UV stress

Experiment 4: HA

A last study was performed in analogy with above experiment 3. In this case, however, CutiGuard CLR™'s activity on HA production was evaluated. Method of measurement: Hyaluronan ELISA, DY3614-05, (Bio-techne (R&D)), 450/570 nm. Results obtained on skin models which were non-treated and non-stressed at day 2 were set at 100%.

Results

A somewhat surprising result was obtained on the skin models which were not stressed and not treated with CutiGuard CLR™. The HA expression in this control was elevated. The skin models which had been under UV stress, however, showed strongly reduced HA expression. In both cases, though, it could be clearly shown that CutiGuard CLR™ could strongly help elevate the production of HA in the full skin models (Fig. 13). Strikingly, in both cases the HA expression was clearly higher than the baseline expression obtained at day 2.

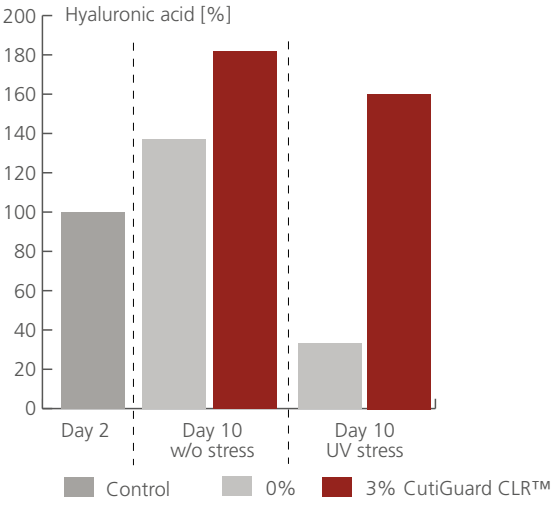

Fig. 13: Influence on expression of Hyaluronic acid, with and w/o UV stress

EFFICACY STUDIES – *in vivo* assays

From the results obtained in the numerous *in vitro* studies, it can be concluded that CutiGuard CLR™ has dual relevant activities on skin aging. On the one hand it acts on the most important cellular processes in the skin which induce skin aging, on the other CutiGuard CLR™ supports skin cells, even under skin aging–relevant stress, and in producing molecules such as Hyaluronic acid and Procollagen I. These activities make CutiGuard CLR™ extremely relevant for the modern anti-aging skincare consumer who, most of all, is interested in slowing down the skin aging processes. It is, however, also important to provide them with skincare products which help them combat skin aging features as soon as they become visible.

In order to prove that CutiGuard CLR™ acts on the most relevant visible signs of skin aging, a number of *in vivo* studies were performed. In these studies, the focus was on the three most important features: wrinkles (especially in the eye area), skin firmness and elasticity (related to skin sagging) and homogeneity, i.e., evenness of skin coloration (which can originate from uneven pigmentation, but also phenomena like Collagen loss, Collagen glycation, inflammation, etc.).

The volunteers included in the studies were between 36 and 50 y/o, Caucasian, female, and showed fine to medium wrinkles in eye area. They were asked to apply the test products (verum, containing 3% CutiGuard CLR™ and placebo, identical formulation, without CutiGuard CLR™) twice daily (mornings and evenings).

One study was performed on 18 volunteers over a period of 28 days, where the activity of the test products was assessed on the inner forearms. In this study, the mechanical properties of skin were measured by making use of a cutometer. Further details about this study are given below.

Other studies were performed on 32 volunteers. One half of the face (hemiface) was treated with verum and the other half with placebo. The studies were double-blind, both the volunteers and the contract research laboratory which performed the studies had no knowledge about the nature of the formulations, i.e., no knowledge about which formulation contained which ingredients. Studies on the face were performed during 28 to 42 days.

Important other specifics of the studies were related to the need of a study model where skin went through a period of severe stresses with an impact on skin aging. It was decided to perform the study in the fall (September, October, November) in a city in the north of Europe.

During this period of time, the weather deteriorates rather dramatically. In September the weather can still be pleasant, whereas during October and November the weather becomes considerably worse and the outside temperatures lower. The strong deterioration of the outside circumstances is a stress that alone accelerates skin aging. Northern Europeans are inclined to move more indoors to warmer but drier environments, which adds further stress to skin. Northern European city dwellers are described to suffer from increased psychological stress, which can be linked to elevated bodily cortisol levels during this time of year. This is another factor that accelerates skin aging.

Another important specific of the study included the skincare regime of the study volunteers. It was deliberately decided to allow the volunteers to continue using their anti-aging skincare products until shortly (12 hours) before the start of the study and not implement a so-called wash-out phase (a longer period of time where the volunteers are not allowed to use any skincare products). A total of 91% used anti-aging day care products and 50% anti-aging night care products before the study. The products used belonged to such brands as Guerlain, L'Oreal Revitalift, Filorga and Clarins.

The above was decided because anti-aging skincare consumers normally use these products daily or twice daily. Their skin tends to be taken care of well. In case they switch to another brand or product, this new product needs to outperform the product(s) they had been using thus far. The aim was to prove that CutiGuard CLR™ helps in outperforming the anti-aging skincare products they used until 12 hours before the start of the study.

Skin firmness and elasticity

The firmness and elasticity of skin are strongly related to skin sagging, one of the main visible features of skin aging. When skin loses its firmness and elasticity, it starts to sag. Firmness and elasticity of the skin can be measured with a cutometer. A cutometer is a so-called suction chamber device that measures the vertical deformation of the skin when it is pulled by a controlled vacuum into the probe of the cutometer. It also measures relaxation of skin (its return to its original state) after release of the vacuum. Altogether it measures skin deformation over time where the vacuum is applied and released repeatedly. The mechanical/rheological behavior of skin during a study with a cutometer can be graphically described as in figure 14.

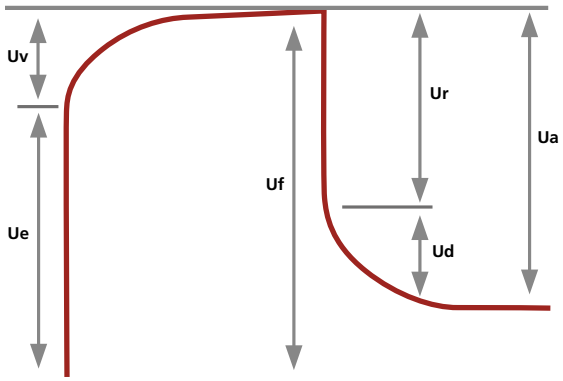

Fig. 14: Skin deformation over time in a cutometer experiment

The cutometer variables which are determined, are:

- Ue: immediate distension
- Uv: delayed distension
- Uf: final deformation
- Ur: immediate retraction
- Ua: total recovery

These variables allow for a mathematical calculation of skin and skin aging–relevant parameters:

- R0 = Uf: correlates inversely with skin firmness.
- R1 = Uf - Ua: a measure for the ability to return fully to its original state after release of the vacuum. Correlates with skin tonicity.
- R2 = Ua / Uf: "overall elasticity" of skin including viscous deformation. Reduction of R2 correlates with skin aging.
- R5 = Ur / Ue: "net elasticity" without viscous deformation. Reduction of R5 correlates strongly with skin aging.
- R7 = Ur / Uf: "biological elasticity." Reduction of R7 correlates very strongly with skin aging.

The study was performed on the inner side of the forearms on 18 volunteers with a Cutometer MPA 580 (Courage & Khazaka, Germany). The results obtained with placebo were set at 0%.

Results

Compared to the placebo, the formulation containing 3% CutiGuard CLR™ improved all parameters related to skin firmness and elasticity (Fig. 15). Especially the parameter which correlates most strongly with skin aging, R7, but also R5, were strongly impacted by CutiGuard CLR™.

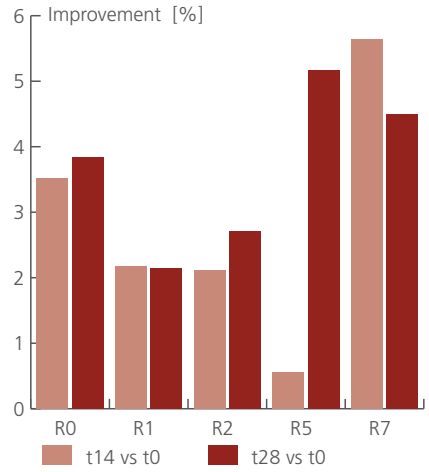

Fig. 15: Improvement of cutometer parameters versus placebo

Density of lines and wrinkles

AEVA-HE 3D Imaging (Eotech SAS, France) uses fringe projection to analyze the topography of the skin surface and allows for visualization of the results (as shown in Fig. 16). This technology enables the analysis of overdensity of line and wrinkles on the face. In this case, density is defined as the surface occupied by fine lines and folds divided by the total surface area (in this case the hemi-face). Referring to figure 16, the density of lines and wrinkles equals the surface area of the colored areas on the hemi-face divided by the total surface area of the hemi-face, i.e., the surface area of the colored lines and the gray area combined.

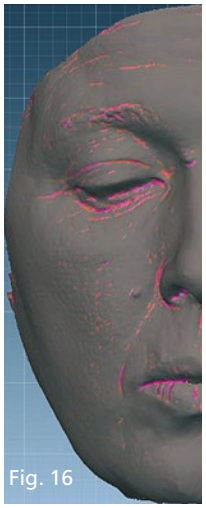

Fig. 16: Illustration of AEVA-HE 3D-imaging technology for determination of density of lines and wrinkles

Results

Over the period of 4 weeks it could be clearly shown that the placebo did not have any effect on the density of lines and wrinkles, whereas the formulation with 3% CutiGuard CLR™ did (Fig. 17). After 14 days a result was already visible. These results indicate that, even though the skin of the volunteers was well cared for at the beginning of the study and the skin was under increased stress, CutiGuard CLR™ still was able to improve the skin.

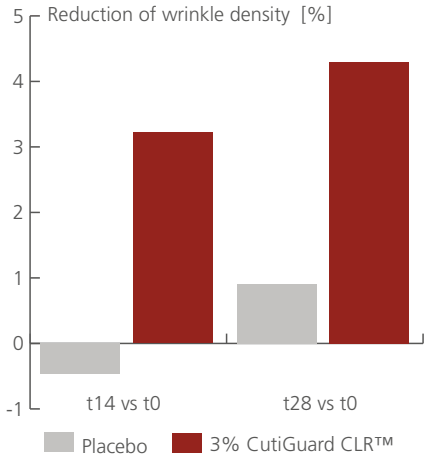

Fig. 17: Influence on wrinkle density, hemi face

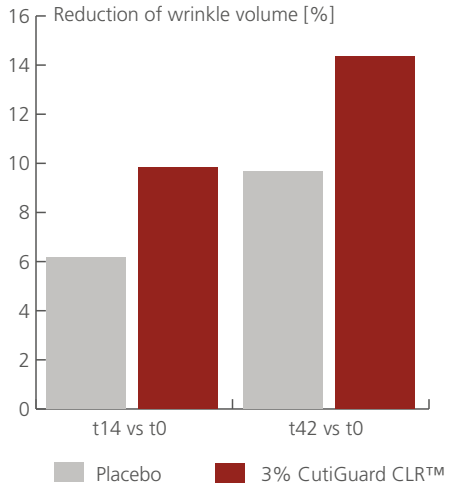

Fig. 18: Influence on wrinkle volume, crow's feet area

Fine lines and wrinkles in the eye area

Further in-depth analysis of the effect of the test products on wrinkles in the eye area was performed photographically using the ColorFace® technology of Newtone Technologies, France. This technology includes 2D high-resolution standardized photography. For the analysis of wrinkle characteristics, a standard 60 lighting filter (STD60) was used, cropped images with segmentation. From the standardized photographs and with the technologies of Newtone, wrinkle volume (multiplication of pixels (surface area) and pixels (depth)), length (total number of pixels in the morphological skeleton of the area) and surface area (total number of pixels in the area) were determined. Wrinkle volume, length and surface area of skin at t = 0 (beginning of the study) were set at 0%.

Results

Interestingly, both placebo and verum with 3% CutiGuard CLR™ had a positive influence on all three parameters (Fig. 18, 19 and 20). CutiGuard CLR™ was able to clearly improve the effect of the placebo formulation, however. As the first signs of skin wrinkles mostly appear in the eye (crow's feet) area and, at t = 0, was already well cared for, the results obtained in this study show the great relevance of CutiGuard CLR™ in supporting the skin against one of the most important early signs of skin aging.

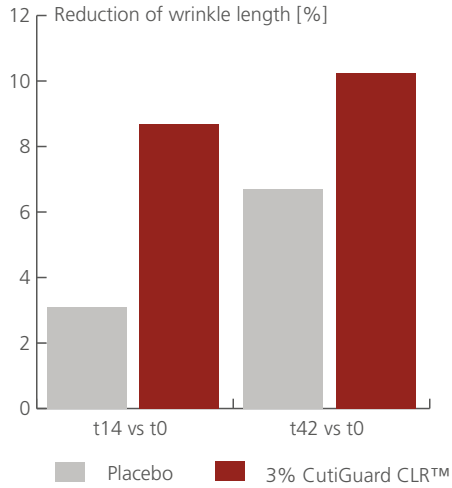

Fig. 19: Influence on wrinkle length, crow's feet area

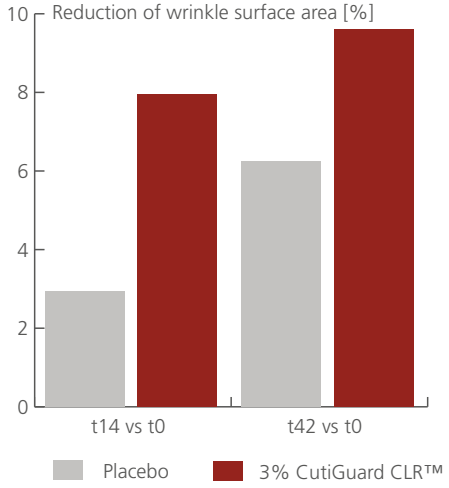

Fig. 20: Influence on surface area, crow's feet area

Some examples of individual results from this study are shown in figures 21 and 22.

In both cases it is clearly visible that CutiGuard CLR™ not only affects wrinkles but makes the overall structure/ topography, i.e., skin relief, of skin finer and smoother.

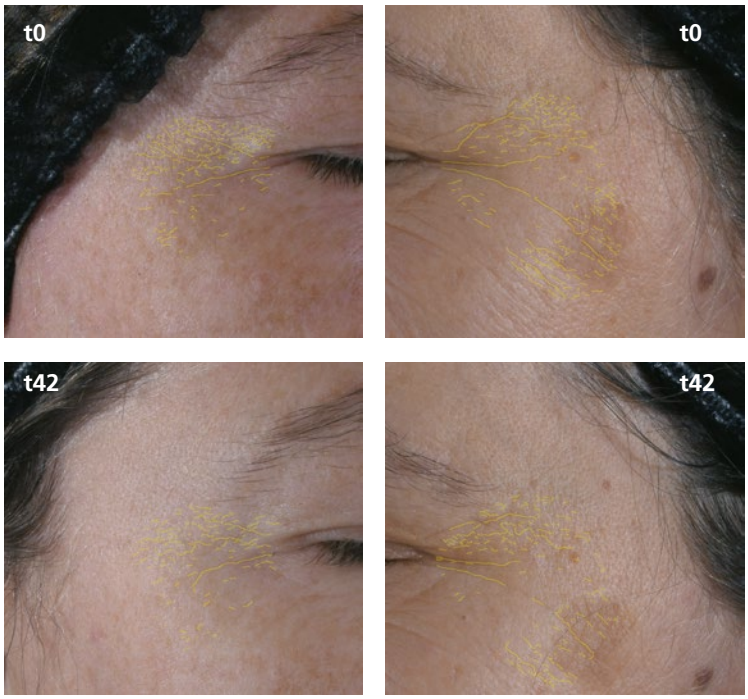

Fig. 21: Individual results obtained on volunteer 11

Fig. 22: Individual results obtained on volunteer 22

Skin relief

In order to further analyze the effect of CutiGuard CLR™ on the smoothness of skin surface, an in-depth analysis was performed using AEVA-HE 3D Imaging System (Eotech SAS, France), Fringe projection. This measurement took place on the cheeks. Results obtained with placebo were set at 0%.

Results

At all timepoints during the study, it could indeed be shown that, compared to placebo, the verum containing 3% CutiGuard CLR™ led to a finer and smoother skin surface (Fig. 23).

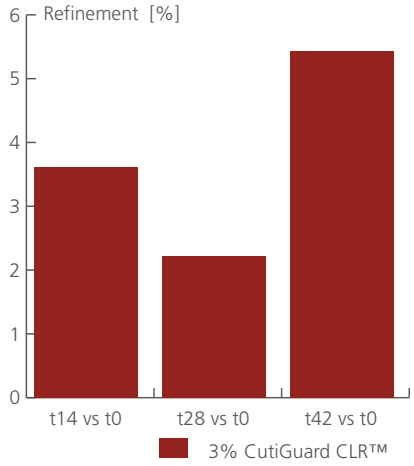

Fig. 23: Influence on skin relief versus placebo

Skin color evenness

Next to skin sagging and wrinkling, the third most important factor in "skin looking old" is the evenness of skin coloration in the face. In fact, a study performed by Chanel (A. Pocheron et al., "Influence of skin ageing features on Chinese women's perception of facial age and attractiveness," Int. J. Cosmet. Sci. 2014 Aug;36(4):312–20), showed that skin color evenness might be an even more important factor than wrinkles and skin sagging.

Skin color evenness is, therefore, an extremely important parameter for modern anti-aging skincare products. The effect of CutiGuard CLR™ on skin color evenness was assessed with the ColorFace® technology of Newtone Technologies, France. This technology includes 2D high-resolution standardized photography. For the analysis of skin color evenness, a cross-polarized filter (CP) was utilized.

In the context of the technology used, skin color evenness is defined as the degree of color (L*, a* and b* values) dispersion within the region of skin which is of interest. The parameter of interest, in this case, is called H76. H76 represents the mean value of color differences between each pixel in the region of interest, in other words: H76 equals the standard deviation of the quantified colors of all the pixels in the region of interest. When skin color is even, H76 reaches zero. The more uneven the skin coloration, the higher H76 will become.

The values of H76 obtained in the studies were analyzed and then mirrored to the effect of a makeup product. The reasoning behind this was that makeup can safely be considered a benchmark in making the skin color even. It does not need further explanation that an active ingredient acting on the biology of skin cannot compete with the pigments used in makeup cosmetics. It is, however, relevant to make this comparison as the use of makeup cosmetics is on the decline, whereas the consumer still feels the need to have an even skin complexion. On the basis of a publication by C. Batres et al. ("Cosmetics increase skin evenness: Evidence from perceptual and physical measures," Skin. Res. Technol., 2019 Sep;25(5):672–76) a comparison was made between the results obtained with CutiGuard CLR™ and those with a makeup cosmetic. The effect on H76 of the makeup product was set at 100%.

Results

Strikingly, after 6 weeks the verum containing 3% CutiGuard CLR™ was able to reach 27% of the effect of the makeup cosmetic product (Fig. 24). The effect of the placebo formulation did not further improve after 28 days of application of the products, whereas the effect of the verum did.

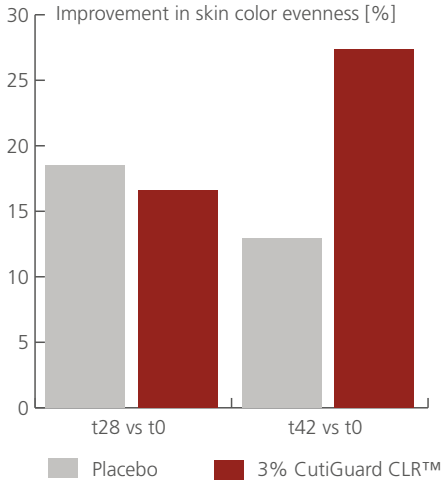

Fig. 24: Influence on skin color evenness in comparison to a makeup product

CONCLUSIONS

The average age of the anti-aging skincare consumer is shifting to an ever-younger age. With that, the expectations of anti-aging skincare products have shifted as well. Now, more than ever, consumers are proactive when it comes to their aging skin. They realize that it is indeed better to prevent than to cure. Consumer concerns about skin aging have remained the same though. They are mostly concerned about the visible signs of skin aging, i.e., "looking old."

CutiGuard CLR™ was developed to fulfil the needs of the aging skincare consumer. It reduces the number of senescent cells, a type of cell which can be considered to be a driver of skin aging. Through this activity it reduces the negative impact of senescent cells. CutiGuard CLR™ reduces the expression of senescence associated Interleukin-6 and -8 as well as the destructive protease Pro-MMP-1. It also reduces the expression of HMGB1 which also plays an important role in driving the skin aging processes. Under aging-related cellular stress, CutiGuard CLR™ was still able to improve the production of Hyaluronic acid and Procollagen I.

The most important visible signs of skin aging are skin wrinkling, loss of firmness and sagging and uneven skin coloration. These three aspects were the subject of the *in vivo* studies which were performed with CutiGuard CLR™. It could indeed be shown that it improves these aspects. Altogether, CutiGuard CLR™ can slow down the skin aging process and acts on the most important skin characteristics of "looking old." With that, CutiGuard CLR™ can be seen as an "all-in-one" anti-aging skincare ingredient for the modern aging skincare consumer.

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CutiGuard CLR™ 01_e

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